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Cell cycle control by the thyroid hormone in neuroblastoma cells

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Abstract

The thyroid hormone (T3) blocks proliferation and induces differentiation of neuroblastoma N2a-β cells that overexpress the β1 isoform of the T3 receptor. An element in the region responsible for premature termination of transcription mediates a rapid repression of c-*myc* gene expression by T3. The hormone also causes a decrease of cyclin D1 gene transcription, and is able to antagonize the activation of the cyclin D1 promoter by Ras. In addition, a strong and sustained increase of the levels of the cyclin kinase inhibitor (CKI) $p27^{Kip1}$ are found in T3-treated cells. The increased levels of $p27^{Kip1}$ lead to a marked inhibition of the kinase activity of the cyclin–CDK2 complexes. As a consequence of these changes, retinoblastoma proteins are hypophosphorylated in T3-treated N2a-β cells, and progression through the restriction point in the cell cycle is blocked.
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1. Introduction

Although the thyroid hormone (triiodothyronine, T3) is essential for normal brain development, the specific mechanisms by which this hormone controls neuronal proliferation and differentiation are not yet well defined.

Different signals that arrest cell growth and induce differentiation suppress the expression of the c-*myc* gene, an important player in cell cycle progression (Grandori et al., 2000). On the other hand, inactivation by phosphorylation of retinoblastoma proteins (pRB), catalyzed by the cyclin-dependent kinases (CDKs), is required for progression through the restriction point in the cell cycle (Sherr, 1996). The activities of the CDKs are in turn regulated by different mechanisms including interaction with cyclins and association with a group of inhibitory proteins named cyclin-kinase inhibitors or CKIs (Morgan, 1995).

T3 blocks proliferation in G0/G1 and induces differentiation of murine neuroblastoma N2a-β cells that overexpress the thyroid hormone receptor β1 isoform (Lebel et al., 1994). To gain some insights into the mechanisms by which T3 regulates neuronal cell growth and differentiation, we have analyzed the effect of this hormone on the expression and activity of cell cycle-regulating molecules in N2a-β cells. Our results show that

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incubation with T3 leads to a coordinated regulation of the expression of several genes that play a key role in cell cycle control including a rapid down-regulation of the c-*myc* gene, a decrease of cyclin D1 transcription, and an induction of the CKI $p27^{Kip1}$. As a consequence, pRb are hypophosphorylated and growth of T3-treated N2a-β cells is arrested.

2. Methods

2.1. RNA extraction and hybridization

Northern blot analysis was carried out (Perez-Juste and Aranda, 1999) with labeled mouse cDNA probes for c-myc, cyclin D1 or $p27^{Kip1}$ and total RNA from N2a-β cells, a kind gift of J. Puymirat.

2.2. Immunoblotting, immunoprecipitation and kinase assay

Western blottings were carried out with cell proteins and antisera anti-c-myc, anti-cyclin D1, anti-$p27^{Kip1}$, anti-p130 or anti-Rb. Immunoprecipitates from cell lysates incubated with anti-CDK2 antibody agarose conjugated were separated by SDS-PAGE and revealed with the anti-p27 antibody. To measure kinase activity, ^{32}P-labeled histone H1 was determined after the incubation of the immunoprecipitates with histone H1 and [g-^{32}P]ATP (Perez-Juste and Aranda, 1999).

2.3. Transient transfections

The cells were transfected with calcium phosphate with 5 μg of reporter plasmids containing a fragment of the murine c-myc gene fused to chloramphenicol acetyltransferase gene (Perez-Juste et al., 2000) or with a plasmid in which cyclin D1 promoter was ligated to the luciferase gene. The cyclin D1 plasmid was transfected alone or in combination with 100 ng of a vector encoding oncogenic Ras^{val12}.

3. Results

T3 causes a rapid decrease of c-*myc* transcripts in N2a-β cells. The levels of c-*myc* transcripts remained low for at least 72 h (Fig. 1A). As shown in Fig. 1B, incubation with T3 causes a rapid repression of the activity of a plasmid containing sequences +137/+516 of the c-*myc* gene. This region contains the sequences responsible for premature termination of transcription of the c-*myc* gene (Ryan and Birnie, 1996). A negative thyroid hormone response element (nTRE) that maps precisely within the region of the polymerase II pausing and release, is adjacent to the binding site for the transcriptional repressor CTCF (data not shown). As shown in Fig. 1C, the functionality of the TRE seems to depend on its position with respect to the transcription initiation site. T3 decreased the activity of a reporter plasmid in which a c-*myc* fragment containing the nTRE and the CTCF binding site was cloned downstream of a heterologous promoter.

The levels of cyclin D1, another important component of cell cycle progression, were also

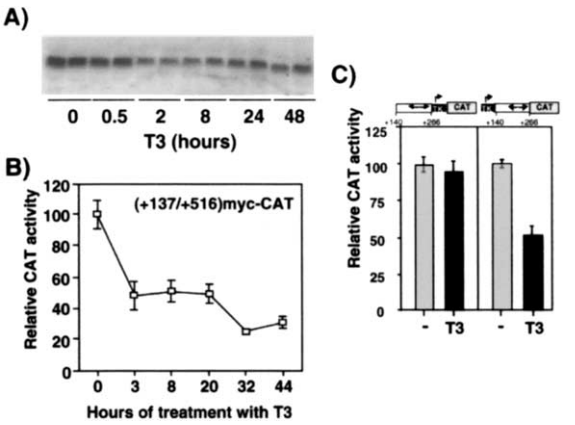

Fig. 1. T3 decreases c-*myc* gene expression in N2a-β cells. (A) c-*myc* mRNA levels were determined in cells incubated for the indicated time periods in the presence or absence of 5 nM T3. (B) CAT activity of a construct containing the c-*myc* mouse promoter fragment +137/+516 in control cells and in T3-treated cells. (C) The activity of the TRE depends on its location. The region +140 to +266 of the c-*myc* gene was cloned either upstream or downstream of the TK promoter construct and CAT activity was determined after 24 h in control and T3 treated cells.

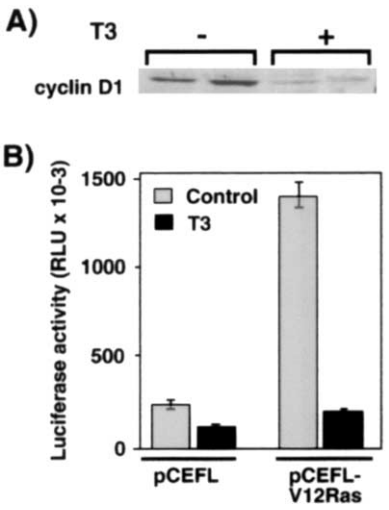

Fig. 2. T3 represses cyclin D1 expression. (A) Protein levels of cyclin D1 in control and T3-treated N2a-β cells after 48 h. (B) Activity of a reporter plasmid containing the $-1720/+139$ fragment of the cyclin D1 promoter in N2a-β cells cotransfected with a construct expressing V12 H-Ras or the corresponding empty vector (pCEFL). Luciferase activity was determined after 48 h of incubation in the presence or absence of 5 nM T3.

strongly reduced after T3 treatment (Fig. 2A). The activity of the cyclin D1 promoter is activated by proliferative signals which activate the Ras oncoproteins (Gille and Downward, 1999) and as shown in Fig. 2B, T3 was able not only to reduce basal promoter activity, but also to markedly inhibit the transcriptional response to Ras.

Since CKIs are targeted by different growth-inhibitory and differentiation signals, the possibility that T3 could induce the expression of CKIs was also analyzed. As shown in Fig. 3A and Fig. 3B, transcripts for the CKI $p27^{Kip1}$ as well as $p27^{Kip1}$ protein levels were induced in N2a-β cells incubated with T3. In pulse-chase experiments, we found that the half-life of the $p27^{Kip1}$ protein was much shorter in the untreated N2a-β cells than in the cells treated with T3 (data not shown). Thus, protein stabilization clearly contributes to the accumulation of $p27^{Kip1}$ protein observed in the cells incubated with T3.

CDK inhibitors are thought to prevent cell proliferation by interaction with cyclin-CDK complexes. As shown in Fig. 3B, the amount of $p27^{Kip1}$ precipitated with anti-CDK2 antibody increased significantly in T3-treated cells. Consequently, CDK2-associated kinase activity was strongly reduced by T3 in N2a-β cells. (Fig. 3C)

The effects of T3 on pRb family proteins was also investigated. Fig. 3D shows that T3 produced a significant accumulation of unphosphorylated

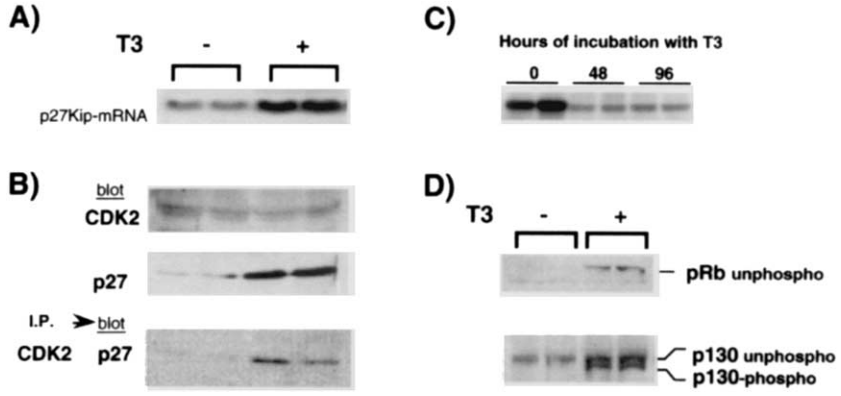

Fig. 3. T3 increases $p27^{kip1}$ expression, reduces CDK2 activity and causes pRB hypophosphorylation. (A) $p27^{kip1}$ mRNA levels in N2a-β incubated with or without 5 nM T3 for 24 h. (B) Association of $p27^{kip1}$ with CDK2 in T3-treated or control N2a-β cells. After incubation with or without 5 nM T3 total levels of CDK2 and $p27^{kip}$ were determined by immunoblotting (blot) with the corresponding antibodies. In the bottom panel the cell lysates were immunoprecipitated (I.P) with the anti-CDK2 antibody, followed by immunoblot analysis of precipitates with the $p27^{kip}$ antibody (blot). H1 kinase activity associated with CDK2 was determined in immunoprecipitates from N2a-β cells treated with or without 5 nM T3 for the indicated time periods. (C) Levels of hypophosphorylated pRb family proteins were analyzed by inmunoblotting in control and T3-treated cells after 48 h.

pRb. In addition, the hormone induced a shift from the slower migrating hyperphosphorylated p130 found in control N2a-β cells to their faster migrating hypophosphorylated forms.

4. Discussion

Our results show that T3-mediated growth arrest and differentiation of neuroblastoma cells is associated with hypophosphorylation of the retinoblastoma proteins. Under these conditions they associate with E2F/DP factors and repress transcription of target genes required for progression through the restriction point in the cell cycle (Weinberg, 1996).

It has been reported that inhibition of CDK activity and pRb phosphorylation are major determinants for neuronal differentiation (Kranenburg et al., 1995). In agreement with the important role of CKIs on neuronal differentiation, we find that T3 induces a strong and sustained increase of the mRNA and protein levels of $p27^{Kip1}$, and leads to a strong reduction of the CDK2 activity.

Cyclin D-dependent kinases also induce pRb hyperphosphorylation (Sherr, 1996). Since T3 reduces cyclin D1 expression, this decrease, as well as the induction of $p27^{Kip1}$ and the consequent inhibition of CDK2 activity, might contribute to maintain pRb family proteins in their hypophosphorylated state.

$p27^{Kip1}$ and the c-*myc* proto-oncogene have generally opposite roles in cell growth control, and c-Myc antagonizes the growth arrest induced by $p27^{Kip1}$ (Henriksson and Lüstcher, 1996). Therefore, it was not surprising that T3 reduced *c-myc* gene expression in N2a-β cells. Suppression of c-Myc levels by T3, assuredly contributes to growth arrest and differentiation of neuroblastoma cells.

In summary, our results indicate that T3 coordinately regulates the expression of several genes which play a key role in cell cycle control and differentiation of neuronal cells. These changes do not allow progression through the restriction point in the cell cycle, and provide a mechanism to explain the crucial effects of thyroid hormones on neuroblastoma cell growth and differentiation.

Acknowledgements

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