Retinoic Acid Induces p27^Kip1 Nuclear Accumulation by Modulating Its Phosphorylation

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Abstract

All-trans-retinoic acid (ATRA), the most biologically active metabolite of vitamin A, controls cell proliferation, apoptosis, and differentiation depending on the cellular context. These activities point to ATRA as a candidate for cancer therapy. A pivotal effect of the molecule is the modulation of p27^Kip1, a cyclin-dependent kinase (CDK) inhibitor (CDKI). Here, we investigate the mechanisms by which ATRA regulates p27^Kip1 level in L-AN-5, a neuroblastoma cell line. When added to the cells, ATRA causes a rapid nuclear increase of p27^Kip1, which clearly precedes growth arrest. The early buildup is not due to impairment of the CDKI degradation, in contrast to previous observations. Particularly, we did not detect the down-regulation of Skp2 and Cks1, two proteins involved in the nuclear ubiquitin-dependent p27^Kip1 removal. Moreover, the regulation of Skp2 and Cks1, two proteins involved in the observations. Particularly, we did not detect the down-regulation of Skp2 and Cks1, two proteins involved in the nuclear ubiquitin-dependent p27^Kip1 removal. Furthermore, the characterization of CDKI morphogenesis does not impair the CDKI nuclear export and does not cause CDK2 relocalization. The characterization of CDKI isoforms by two-dimensional PAGE-immunoblotting showed that ATRA induces an early nuclear up-regulation of mono-phosphorylated p27^Kip1. Immunologic studies established that this isoform corresponds to p27^Kip1 phosphorylated on S10. The buildup of phospho(S10)p27^Kip1 precedes the CDKI accumulation and increases its half-life. Finally, ATRA-treated normal LAN-5 extracts showed an enhanced capability of phosphorylating p27^Kip1 on S10, thus explaining the nuclear up-regulation of the isoform. In conclusion, our data suggest a novel mechanism of ATRA antiproliferative activity, in which the morphogen rapidly up-regulates a nuclear kinase activity that phosphorylates p27^Kip1 on S10. In turn, this event causes the stabilization of p27^Kip1 and its accumulation in the nuclear compartment. (Cancer Res 2006; 66(8): 4240-8)

Introduction

All-trans-retinoic acid (ATRA), the biologically active form of vitamin A, plays a pivotal role in early embryonic development and in the maturation of several tissues and organs, including the nervous system (1, 2). In vitro, ATRA regulates the transition from proliferating precursor cell to postmitotic differentiated cell, being many examples of distinct cell types whose differentiation is under its control (3–7). An in vivo corollary of these properties has been the successful use of the molecule in the treatment of some human cancers, such as acute promyelocytic leukemia and neuroblastoma.

The process of terminal differentiation is strictly coupled to growth arrest in the G0-G1 phase of the cell division cycle. Cell cycle progression depends on the activity of cyclin-dependent kinases (CDK), which in turn can be regulated by cyclins and CDK inhibitors (CDKI). ATRA-dependent growth impairment is associated with the modulation of several components of the cell cycle engine, particularly the down-regulation of cyclins and the up-regulation of p27^Kip1 (8–12).

p27^Kip1 is a critical regulator of G1 progression because it interacts with and inhibits cyclin E-CDK2 complex (13, 14). The CDKI also modulates, in mid-G1, the assembly and nuclear import of cyclin D-CDK4/6 complexes (15). Then, it rapidly decreases during G1-S phase transition. p27^Kip1 cellular content is regulated by intricate post-translation mechanisms and proteasome degradation (16–18). Several pieces of evidence suggest that p27^Kip1 degradation is controlled by two distinct ubiquitination processes that occur in the nuclear and cytosolic compartments. Nuclear ubiquitin-dependent p27^Kip1 degradation requires a previous phosphorylation on T187 catalyzed by active CDK2 in late G1 and S phases (19–22). T187 phosphorylation permits p27^Kip1 recognition by its E3 ligase of SCF-type complex formed by Skp1, Cul1, Skp2, Roc1, and Cks1 (23–27). Conversely, the cytosolic ubiquitination occurs in a manner independent on T187 phosphorylation and is required in G0 and early G1 phases (28, 29). The E3 complex responsible for the cytosolic removal (Kip1 ubiquitination-promoting complex) has been identified and characterized recently (30, 31).

The occurrence of two distinct proteolytic systems point to the CDKI nuclear localization as a pivotal mechanism for controlling p27^Kip1 level and function. Nuclear degradation requires the import of the protein, which has been proposed to involve the interaction of p27^Kip1 with the nuclear pore protein Nup50/Nup60 (32). Phosphorylation on T187 or T198, due to activated AKT/protein kinase B, hampers nuclear import (33, 34) and decreases p27^Kip1 nuclear content, thus resulting in CDK2 activation and cellular proliferation. The nuclear export of the protein requires a complex series of events and several proteins. An initial step has been reported to be the phosphorylation on S10, which allows p27^Kip1 binding to the carrier protein CRM1 (35, 36). Additional experiments suggest that Jun activation domain-binding protein 1 (Jab1) participats to the nuclear export of p27^Kip1 by functioning as an assembly factor (37). However, recently, the phosphorylation on S10 as a pivotal step for nuclear export of p27^Kip1 has been questioned seriously (38), and the postsynthetic modification seems to increase solely the CDKI stability (39).

The kinase (or kinases) catalyzing p27^Kip1 phosphorylation on S10 has not been definitely identified (35, 36, 40, 41). Two enzymes

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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have been proposed to catalyze the reaction. Human kinase interacting stathmin (hKIS) has been reported to phosphorylate p27Kip1 in response to growth stimuli during the G1→S transition (40). In this case, the reaction commits the CDKI to exit from nuclei and to subsequent cytosolic degradation. The other enzyme (i.e., Mirk/dyk1B kinase) is maximally active in G0 and thus might be responsible for the phosphorylation of the CDKI during this phase (41). However, recent data questioned the phosphorylation of p27Kip1 in G0 (38) and showed that this modification occurs only during G1→G0 transition. In conclusion, although the majority of phosphorylated p27Kip1 is modified on S10, the precise function of the phosphorylation and the kinase(s) catalyzing the reaction has not been identified definitely.

Because of the importance of p27Kip1 up-regulation in the ATRA activity, we decided to investigate the molecular mechanism(s) by which retinoic acid induces p27Kip1 increase. The obtained results point to p27Kip1 phosphorylation on S10 as a major target of ATRA action.

**Materials and Methods**

**Materials.** ATRA was from Sigma Chemical Co. (St. Louis, MO), AM-580, methotrene acid, U0126 [mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitors], calphostin C [protein kinase C (PKC) inhibitor], myristoylated protein kinase inhibitor (PKI; 14-22 amide) [protein kinase A (PKA) inhibitor], and staurosporine were furnished by BIOMOL International, LP (Plymouth Meeting, PA). SH5 (AKT inhibitor) was from Alexis Biochemicals (San Diego, CA). Recombinant cyclic AMP–dependent protein kinase (PKA; catalytic subunit) and λ protein phosphatase were obtained from Cell Signaling Technology (Beverly, MA). The mouse p27Kip1 coding sequence cloned into pET21a expression vector and the human wild-type (WT) p27Kip1 and the S10A mutant p27Kip1 coding sequence, cloned into the pcDNA3 plasmid, were purchased from Zymed Laboratories, Inc. (South San Francisco, CA). mAbs against phosphothreonine, phosphoserine, and phospho(T187)p27Kip1 were purchased from Zymed Laboratories, Inc. (South San Francisco, CA). β-Actin and β-tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA).

**Antibodies.** Monoclonal antibodies (mAb) to p27Kip1 were from BD Transduction Laboratories (Franklin Lakes, NJ). mAb against Skp2 and polyclonal antibodies against phosphothreonine, phosphoserine, and phospho(T187)p27Kip1 were purchased from Zymed Laboratories, Inc. (South San Francisco, CA). mAbs against phosphothreonine and phospho(threonine) were furnished by Alexis Biochemicals. Polyclonal antibodies to p27Kip1(C19), phospho(S10)p27Kip1, Skp2, Cks1, CDK2, and histone deacetylase 1 (HDAC1) were from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell lines and treatments.** The source of the cell lines used and their culture conditions were described by Borriello et al. (10). All the treatments were done on cells plated the day before and reaching 60% to 70% confluence.

**Cellular fractionation, p27Kip1 degradation, and p27Kip1 kinase assay.** Nuclear and cytosolic extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL). Extracts obtained by this method were tested for cross-contamination using antibodies against HDAC1 protein as nuclear marker. The other aliquot was treated similarly, except that the phosphatase was not added, and the reaction was incubated at 30°C for 30 minutes. The other aliquot was treated similarly, except that the phosphatase was not added. The control and phosphatase-treated samples were finally processed as described for the two-dimensional analysis.

Recombinant WT human p27Kip1 (60 ng) was immunoprecipitated by anti-p27Kip1 polyclonal antibodies immobilized onto protein A-agarose. The immunoprecipitate was divided in two aliquots. One aliquot was resuspended in 50 µL phosphate-buffered saline (PBS) and treated with λ protein phosphatase (400 units) and another aliquot was treated similarly, except that the phosphatase was not added. The reaction was incubated at 30°C for 30 minutes. The mixture was separated by two-dimensional PAGE and blotted on a nitrocellulose membrane. The blot was firstly analyzed by autoradiography and then subjected to Western blotting.

**Results**

**ATRA induces a Skp2-independent p27Kip1 accumulation.** It has been reported previously by us and others (9–11) that the treatment of neuroblastoma cell lines with ATRA induces cell differentiation and inhibition of growth (Fig. 1; data not shown). The antiproliferative effect seems to be due to the up-regulation of p27Kip1 (Fig. 1B) and the down-regulation of CDK2 activity (data not reported; refs. 10–12). Importantly, morphologic changes (i.e., cell elongation and dendrites appearance) and p27Kip1 increase are observable after 10 to 12 hours and precede the growth arrest, which becomes evident only after 24 to 48 hours of treatment.

The increase of p27Kip1 level has been correlated to its degradation impairment. Indeed, we and others (10–12) observed that cell extracts, prepared from 24- and 48-hour ATRA-treated cells, degrade p27Kip1 less efficiently than control extracts. However, we observed that total and nuclear extracts of LAN-5 cells, cultured for 12 hours with or without ATRA, degraded the CDKI at an identical rate (Fig. 1C; data not shown). Because the up-regulation of p27Kip1 level is evident in 12 hours ATRA-treated cells (Fig. 1B), a direct link between CDKI increase and impairment of its removal impairment is unlikely.

Then, we compared the time-dependent changes of p27Kip1, Skp2, and Cks1 levels in ATRA-treated cells. A clear up-regulation...
of p27Kip1 was detectable at 8 hours of treatment (Fig. 1D and E), whereas the decrease of Skp2 and Cks1 was visible after 24 hours (Fig. 1D and E). The lack of linkage between Skp2 decrease and p27Kip1 up-regulation was also confirmed in ATRA-treated HeLa cells (Fig. 1F).

ATRA and retinoic acid receptor α-selective agonist increase p27Kip1 in the nuclear compartment but not in the cytosol. As shown in Fig. 2A, after 8 hours of ATRA treatment, the up-regulation of p27Kip1 was evident in the nuclear compartment. Conversely, a small decrease of the cytosolic CDK1 was observable. Then, the protein progressively accumulated in the nucleus, whereas it was constant in the cytosol up to 24 hours (Fig. 2A). Afterward, p27Kip1 levels increased in both the cellular compartments (data not reported). These results were also confirmed by immunofluorescence studies (data not shown).

We also tested the effect of retinoic acid receptor α (RARα; AM-580) and retinoid X receptor (RXR; methoprene acid) agonists on LAN-5 cells. Only the RARα agonist induced morphologic differentiation (Fig. 2B) and impaired cell growth (data not shown). The activity of AM-580 on p27\textsuperscript{Kip1} distribution in the two cellular compartments showed the same pattern observed for ATRA. Conversely, methoprene acid did not up-regulate the CDKI level (Fig. 2C).

Two-dimensional gel electrophoresis analysis of the phosphorylated p27\textsuperscript{Kip1} isoforms. Several mechanisms might explain the increase of p27\textsuperscript{Kip1} in the nuclear compartment. Recently, it has been described in prostate cells that p27\textsuperscript{Kip1} nuclear increase is due to a set of mechanisms that are not fully understood. One of these mechanisms involves the modification of p27\textsuperscript{Kip1} by phosphorylation. Phosphorylation of p27\textsuperscript{Kip1} can affect its stability, nuclear localization, and interaction with other proteins.

Figure 1. Effects of ATRA on LAN-5 phenotype and p27\textsuperscript{Kip1} removal. A, LAN-5 cells were incubated with or without (Con) 5 \(\mu\)mol/L ATRA. Cell morphology was then acquired after 12 hours. B. LAN-5 cells were incubated for the reported times with or without 5 \(\mu\)mol/L ATRA. p27\textsuperscript{Kip1} content was determined by immunoblotting. C, LAN-5 cells were incubated with or without 5 \(\mu\)mol/L ATRA and collected after 12 hours. Nuclear extracts were prepared and incubated with 1 mg recombinant mouse p27\textsuperscript{Kip1} as that with Borriello et al. (10). At the reported times, equal aliquots were withdrawn and analyzed by immunoblotting using antibodies against p27\textsuperscript{Kip1}. D, LAN-5 cells were incubated for the indicated time intervals with or without 5 \(\mu\)mol/L ATRA. The content of Skp2, Cks1, and p27\textsuperscript{Kip1} proteins was determined by immunoblotting. E, intensities of Skp2, Cks1, and p27\textsuperscript{Kip1} bands of (D) were quantified by scanning densitometry and expressed as percentage of the value at time 0. F, HeLa cells were incubated for the indicated times with or without 5 \(\mu\)mol/L ATRA. The content of p27\textsuperscript{Kip1} and Skp2 proteins was determined by immunoblotting.

Figure 2A. Effect of ATRA on LAN-5 phenotype and p27\textsuperscript{Kip1} cellular localization. A, LAN-5 cells were grown with 5 \(\mu\)mol/L ATRA and collected at the indicated time intervals. Then, nuclear and cytosolic extracts were prepared. The contents of p27\textsuperscript{Kip1} and HDAC1 were determined by immunoblotting. HDAC1, a nuclear protein, was used to confirm the purity of the cellular fractions. Top, numbers, fold of p27\textsuperscript{Kip1} variation with respect of time 0. B, LAN-5 cells were incubated with 5 \(\mu\)mol/L ATRA, 5 \(\mu\)mol/L AM-580 (RARα agonist), or 5 \(\mu\)mol/L methoprene acid (MET; a RXR agonist). Cell morphology was then acquired after 12 hours. C, LAN-5 cells were grown with 5 \(\mu\)mol/L AM-580 or 5 \(\mu\)mol/L methoprene acid for 8 hours. Then, nuclear and cytosolic extracts were prepared, and p27\textsuperscript{Kip1} content was determined. D, LAN-5 cells were treated with 5 \(\mu\)mol/L ATRA, 5 \(\mu\)mol/L AM-580, and 5 \(\mu\)mol/L methoprene acid for 8 hours. Then, the nuclear CDK2 was determined by immunoblotting.
to the cytosolic CDK2 relocalization (42). However, as shown in Fig. 2D, ATRA did not affect the subcellular compartmentalization of the kinase.

An additional mechanism might be the impairment of p27<sup>Kip1</sup> nuclear exit. However, as shown in the Supplementary Data, the rate of CDKI exit from the nucleus was not modified by ATRA.

It is well known that changes in the phosphorylation status of proteins strongly influence their localization, metabolism, and function. Thereby, we decided to investigate, by a two-dimensional PAGE/immunoblotting strategy, the CDKI phosphorylated forms occurring in the nuclear and cytosolic compartments before and after ATRA addition.

In preliminary experiments, p27<sup>Kip1</sup> was immunoprecipitated, and its isoforms were resolved by two-dimensional electrophoresis (focusing range, pH 4-7 linear gradient) and then detected by immunoblotting. Under these conditions, multiple forms of p27<sup>Kip1</sup> were distributed within an interval of ~1 pH unit (assuming the linearity of the supplied pH gradient gels; Fig. 3A). This pattern was also observed in all the other cellular models investigated (i.e., peripheral lymphocytes, HT-29, HeLa, CaCo2, and PC12; data not shown). The most basic form of p27<sup>Kip1</sup> (form 0) focused at pH ~6.5, which is close to the calculated isoelectric point of human p27<sup>Kip1</sup> (pH 6.54; as established by using the Compute pl/Mw tool provided by the ExPaSy Web site).

Besides p27<sup>Kip1</sup> form 0, all the other forms (except form 1) focused at positions that roughly correspond to those predicted for the addition to the CDKI of entire numbers of negative charges (computed position scale at Fig. 3A, bottom calculated as in refs. 43, 44). The isoforms were thus numbered according to their position relative to this scale. Considering that phosphate groups produce a one-charge shift below pH 6 (HPO<sub>4</sub>: ref. 44), p27<sup>Kip1</sup> phosphoforms 2, 3, and 4 were expected to contain one, two, or three phosphate groups, respectively. The intermediate spot 1 might be due to a different covalent modification.

Several experiments confirmed our interpretation. First, the form 0 comigrated with recombinant human p27<sup>Kip1</sup> protein (data not shown). Second, the hypothesis that spots 2, 3, and 4 were phosphoproteins was shown by their disappearance after protein phosphatase treatment (Fig. 3B). Importantly, the treatment did not cause the loss of form 1. Third, recombinant p27<sup>Kip1</sup> was in vitro phosphorylated with recombinant activated PKA, and the labeled CDKI was mixed with p27<sup>Kip1</sup> immunoprecipitated from cell extract. As shown in Fig. 3C, only spots 2, 3, and 4 comigrated with the labeled phosphorylated p27<sup>Kip1</sup> forms.

Subsequently, we analyzed by two-dimensional PAGE/immunoblotting the nuclear and cytosolic compartments of LAN-5 cells treated with ATRA at different times.

As shown in Fig. 3D, clear changes in the distribution of the various forms occurred. In particular, the retinoic acid induced the accumulation of monophosphorylated form in the nucleus, whereas the isoform 0 increased in the cytosol. Importantly, these changes occurred at 4 hours and, therefore, before the up-regulation of the protein in the nucleus.

p27<sup>Kip1</sup> phosphoprotein on S10 is activated by ATRA. The ATRA-dependent changes of p27<sup>Kip1</sup> phosphoprotein status prompted us to identify the phosphorylated residue(s) of the nuclear and cytosol CDKI. p27<sup>Kip1</sup> was immunoprecipitated from normal and ATRA-treated cells and subjected to one-dimensional immunoblotting analysis by using anti-phosphoamino acid antibodies. The signals obtained by using anti-phosphoserine antibodies showed significant variations (i.e., an increase in the nucleus after ATRA addition; data not shown). Conversely, the anti-phosphothreonine antisera gave very faint signals almost identical before and after ATRA addition (data not reported).

Because it has been shown that the only phosphorylated serine residue of p27<sup>Kip1</sup> is Ser<sup>10</sup> (38, 39), we tested the capability of several antisera (either commercial or kindly given by several investigators) to specifically recognize p27<sup>Kip1</sup> isoform phosphorylated on Ser<sup>10</sup>. We identified one antiserum that is highly specific according to different criteria. First, the antibodies did not recognize large amount (1 μg) of recombinant nonphosphorylated p27<sup>Kip1</sup> (Fig. 4A). Then, we prepared WT and S10A mutant p27<sup>Kip1</sup> and used the proteins as substrates of activated PKA. As shown in Fig. 4A, the antiserum clearly reacted with in vitro phosphorylated WT p27<sup>Kip1</sup>, whereas it did not recognize phosphorylated S10A mutant p27<sup>Kip1</sup>. Finally, when tested in two-dimensional PAGE/immunoblotting analysis by using anti-phosphoamino acid antisera.
immunoblotting, the antiserum detected only the phosphoforms of the CDKI (see below).

By using the selected antiserum, we showed that ATRA induces the accumulation of phospho(S10)p27^Kip1 after 4 hours (Fig. 4B), a time interval that clearly precedes the increase of nuclear p27^Kip1.

To corroborate our observations using other experimental models, we searched for neuronal-like cells that respond to ATRA with a concomitant increase of p27^Kip1. We found that PC12, a rat pheochromocytoma cell line, treated with ATRA, showed a rapid nuclear up-regulation of p27^Kip1 (Fig. 4C) without a decrease of Skp2 (data not reported). When we analyzed the effect of ATRA by two-dimensional PAGE/immunoblotting, we observed a significant accumulation of the phospho(S10)p27^Kip1 in the nuclei likewise to the pattern observed in LAN-5 cells (Fig. 4D).

Then, we evaluated whether the phosphorylation on S10 is responsible for the accumulation of the CDKI. As shown in Fig. 5A and B, ATRA significantly increases the nuclear p27^Kip1 half-life (t1/2). An identical result was obtained by evaluating the nuclear phospho(S10)p27^Kip1 t1/2 (Fig. 5C and D). These findings corroborate the importance of the phosphorylation on S10 in the stabilization of the CDKI and its accumulation in the nuclear compartment.

Effect of ATRA on protein phosphatases. Two hypotheses might explain the accumulation of phospho(S10)p27^Kip1 after ATRA addition: (a) the inhibition of a protein phosphatase that dephosphorylates the p27^Kip1 phosphoisoform and (b) the activation of a specific p27^Kip1 kinase.

To evaluate the importance of protein phosphatase activities, we incubated LAN-5 cells with okadaic acid or calcyclin A, two powerful inhibitors of protein phosphatase activities, and evaluated the level of p27^Kip1. The compounds neither increased p27^Kip1 level nor changed its cellular distribution (Fig. 5E). Then, we investigated the effect of ATRA on the activity of protein phosphatase 2 (PP2) isozymes (PP2A, PP2B, and PP2C) in nuclear and cytosolic compartments. We did not identify any changes in PP2 activity and distribution (Fig. 5F). Finally, we investigated the content of protein phosphatase 1 (PP1) and PP2Ac (the catalytic subunit) in nuclei or cytosol of ATRA-treated LAN-5 cells. As reported in Fig. 5G, the proteins content and localization was not influenced by ATRA. We concluded that the increase of phospho(S10)p27^Kip1 is not correlated to the modulation of protein phosphatase activity.

Effect of ATRA on p27^Kip1 kinase activity. To further unravel the ATRA effect on p27^Kip1 phosphorylation, we investigated the activity of retinoic acid on the kinases that are known to act on the CDKI [i.e., AKT (33, 34), ERK1/2 (45, 46), and hKIS (40)]. The results reported in the Supplementary Data allowed us to rule out that these kinases are involved in the accumulation of phospho(S10)p27^Kip1.

Because we were unable to identify the nuclear enzyme that phosphorylated the CDKI on Ser10, we decided to use the antiserum against phospho(S10)p27^Kip1 to develop a specific kinase assay. Thus, the recombinant protein was incubated with nuclear extract, and the assay mixtures were analyzed by immunoblotting for either the total CDKI or its S10-phosphorylated isofrom. As shown in Fig. 6A, the addition of nuclear extract strongly increased the signal of phospho(S10)p27^Kip1, whereas the addition of a heat-inactivated extract gave a signal similar to the recombinant protein alone. In addition, the absence of ATP resulted in the inability of the nuclear extract to phosphorylate the CDKI. Importantly, the cytosolic kinase activity was scarce (data not reported). Finally,
recombinant S10A mutant p27Kip1 was not phosphorylated by the nuclear extract (Fig. 6A). When different amounts of nuclear extracts (both from control and ATRA-treated LAN-5 cells) were assayed using this method, we observed a significant increase of kinase activity in the ATRA-treated samples (Fig. 6B and C). Moreover, an identical result was obtained by using a constant amount of nuclear extract and different quantities of the recombinant proteins (Fig. 6D). These experiments clearly indicated that the treatment with ATRA increased the capability of a nuclear kinase(s) to phosphorylate p27Kip1 on S10. Finally, as shown in Fig. 6E, whereas inhibitors of AKT, PKC, PKA, and MEK were without any effect on the p27Kip1 kinase, staurosporine (a nonspecific kinase inhibitor) completely prevented this enzymatic activity.

Discussion

In this study, we investigated the mechanism(s) by which ATRA induces p27Kip1 accumulation. Because the CDKI buildup clearly precedes growth arrest and induces a strong cell cycle block, p27Kip1 has been considered as an effector of retinoic-dependent phenotypes.

We and others (10, 11) reported previously that ATRA-treated cells accumulate p27Kip1 as the consequence of an impaired CDKI removal. Some putative explanations for this effect have been suggested. Dow et al. (47) showed that ATRA causes the down-regulation of Skp2 level and the consequent poor p27Kip1 ubiquitination and degradation. Similarly, Nakamura et al. (11) reported that the impairment of p27Kip1 removal in ATRA-treated cells is due to either Skp2 down-regulation or N-myc-dependent inhibition of p27Kip1 phosphorylation.

However, a detailed investigation allowed us to show that the CDKI increase clearly preceded both Skp2 and Cks1 decrease. Thus, we concluded that the early ATRA-dependent p27Kip1 buildup is Skp2 independent, whereas the late increase of the CDKI (i.e., starting from 24 hours) might be associated to Skp2 down-regulation.

Recently, increasing pieces of evidence show the importance of cellular compartmentalization in the metabolism of several key proteins, including p27Kip1 (33, 34). Accordingly, we discovered that after ATRA addition, p27Kip1 increases solely in the nuclear compartment. An identical p27Kip1 compartmentalization was visible using a RARα agonist that also induces growth arrest and differentiation. Conversely, a RXR agonist is unable to affect either the cell phenotype or the p27Kip1 content.

A detailed biochemical analysis of p27Kip1 post-translational modifications, by a well-standardized two-dimensional SDS PAGE/immunoblotting technique, showed that the p27Kip1 is a phosphoprotein existing in, at least, monophosphorylated, biphosphorylated, and triphosphorylated forms. The finding agrees with previous observations (38, 48). Moreover, a postsynthetic variant of p27Kip1, distinct from the phosphoforms, was evidenced. Such an unknown modification is now under investigation.

In evaluating p27Kip1 two-dimensional analyses, it is to underline that p27Kip1 phosphorylation profiles were different in the distinct cellular compartments. Thus, two-dimensional studies done on
total cellular extracts, as generally reported, might generate erroneous conclusions. Indeed, when we analyzed separately nuclear and cytosolic extracts, we observed that ATRA differently modifies p27\textsuperscript{kip1} phosphorylation profiles. Particularly, in the nucleus, the accumulation of the monophosphorylated form is observable, whereas in the cytosol the unmodified p27\textsuperscript{kip1} increases. Importantly, these changes are evident well before changes in total p27\textsuperscript{kip1} content, indicating that they precede the accumulation of the protein. Thus, the up-regulation of nuclear p27\textsuperscript{kip1} phosphorylation represents a precocious event in ATRA effect on cell cycle.

Then, we identified by immunologic approaches the increased CDKI phosphoisoform as p27\textsuperscript{kip1} phosphorylated on S10. We also showed that the accumulation of nuclear phospho(S10)p27\textsuperscript{kip1} is associated with the CDKI T/2 elongation as also observed by others (38, 39). Thus, we speculated that ATRA increases nuclear p27\textsuperscript{kip1} by up-regulating the amount of phospho(S10)p27\textsuperscript{kip1}.

Although the transfection of vectors encoding proteins modified at specific phosphorylatable amino acid has been frequently used as a key tool to assess the importance of a specific residue, we decided to avoid this strategy. Indeed, as clearly shown by Kotake et al. (38), the technique might severely alter the metabolism and transport of p27\textsuperscript{kip1}, thus making strongly questionable the obtained conclusions. Conversely, we decided to investigate by direct biochemical approaches the correlation between ATRA and p27\textsuperscript{kip1} phosphorylation. A possible explanation for the increase of the phosphorylated p27\textsuperscript{kip1} form is that ATRA causes the down-regulation of protein phosphatase activities or changes of their localization. However, by different approaches, we definitely ruled out these possibilities. Interestingly, the few data available in literature on the ATRA effect on protein phosphatases report either a positive (49) or a negative (50) modulation, suggesting that the activity of retinoic acid on protein phosphatase activities strongly depends on the cellular model.

At the best of our knowledge, few p27\textsuperscript{kip1} kinases have been putatively identified [i.e., AKT (33, 34), ERK1/2 (51), hKIS (40), and Mirk/dyrk1B (41)]. AKT phosphorylates p27\textsuperscript{kip1} on threonine residues (T157 or T198), making unlikely that the enzyme is responsible for p27\textsuperscript{kip1} serine phosphorylation. However, we did experiments that definitely excluded AKT as the enzyme responsible for p27\textsuperscript{kip1} phosphorylation on Ser\textsuperscript{10}.

Up-regulation of ERK1/2 activity causes a p27\textsuperscript{kip1} level decrease by inducing the translocation of the protein from the nucleus to the cytoplasm followed by the CDKI degradation (45, 46). Accordingly, we and others (the present article; refs. 45, 46) showed that the inhibition of the MAPK pathway results in the increase of nuclear p27\textsuperscript{kip1}. Moreover, in accord with Pasquali et al. (52), we observed that ATRA activates ERK1/2 activity, allowing the exclusion of these kinases in the ATRA-dependent p27\textsuperscript{kip1} up-regulation.

Boehm et al. (40) reported that hKIS phosphorylates p27\textsuperscript{kip1} on S10, thus inducing (a) the CDKI cytosolic relocalization and subsequent degradation and (b) the activation of cell proliferation. Conversely, in our model, ATRA causes the nuclear accumulation of p27\textsuperscript{kip1} and the inhibition of cell growth. This finding putatively excludes hKIS as the ATRA-dependent kinase, which phosphorylates p27\textsuperscript{kip1}. Our immunoblotting data, showing that ATRA does not change hKIS protein level, confirm the hypothesis.

At present, only Mirk/dyrk1B kinase fulfills the major requirements needed to explain the ATRA effect. This kinase indeed phosphorylates p27\textsuperscript{kip1} on S10 and increases during the entry in G0 phase (41). However, two features of Mirk/dyrk1B do not fulfill our requisites. First, the distribution of the enzyme is mostly confined to muscle, whereas phospho(S10)p27\textsuperscript{kip1} is ubiquitous (39, 41). Second, the enzyme is strongly active in G0 (41), whereas,
in this phase, a scarce phosphorylation of p27^Kip1 has been recently shown (38).

Because we ruled out the known p27^Kip1 kinases as responsible of the ATRA effect on the CDKI, we developed an in vitro kinase assay for directly evaluating the capacity of retinoic acid to modulate the nuclear synthesis of phosphoS10p27^Kip1. By this method, we evidenced that ATRA increases the p27^Kip1 kinase activity in the nuclear extracts. Such a finding might explain the data obtained by the two-dimensional analysis and the increase of p27^Kip1 phosphorylated in serine evidenced by one-dimensional immunoblotting. ATRA increases the kinase activity when used different amounts of either nuclear extracts or substrate. Moreover, the enzymatic activity was not detectable in the absence of ATP and was completely inhibited by staurosporine, a powerful but not specific kinase inhibitor. Finally, the activity was not due to AKT, MEK, PKC, and PAK as shown by the lack of effect of specific inhibitors. Studies in development are to further characterize the p27^Kip1 kinase activity.

In conclusion, our study shows for the first time that (a) p27^Kip1 increases only after 8 hours of ATRA treatment; (b) the up-regulation occurs only in the nuclear compartment, whereas the cytosolic content remains unmodified up to 24 hours; and (c) the early CDKJ up-regulation is independent on the decrease of Skp2 level and the consequent impairment of p27^Kip1 degradation system. The CDKI accumulation seems due to the stabilization of nuclear p27^Kip1 and is associated to the increase of kinase activity able to phosphorylate the protein on S10.

Finally, our study on p27^Kip1 isoforms indicates the occurrence on the protein of several phosphorylation sites (at least three), which might be contemporaneously modified, and a new postsynthetic modification clearly distinguishable from phosphorylation.

ATRA is able to induce important phenotypic changes in a large number of cell models, including, particularly, cancer cells. The compound is also an important morphogen and has been introduced in the treatment of several human diseases. Our findings, which indicate that retinoic acid modulates the phosphorylation and compartmentalization of pivotal proteins, may have an importance in shedding new light on the mechanisms of action of ATRA and its analogues.

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Retinoic Acid Induces p27<sup>Kip1</sup> Nuclear Accumulation by Modulating Its Phosphorylation

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