Retinoic Acid Induces p27Kip1 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 p27Kip1, a cyclin-dependent kinase (CDK) inhibitor (CDKI). Here, we investigate the mechanisms by which ATRA regulates p27Kip1 level in LAN-5, a neuroblastoma cell line. When added to the cells, ATRA causes a rapid nuclear increase of p27Kip1, 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 downregulation of Skp2 and Cks1, two proteins involved in the nuclear ubiquitin-dependent p27Kip1 removal. Moreover, the morphogen 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 p27Kip1. Immunologic studies established that this isoform corresponds to p27Kip1 phosphorylated on S10. The buildup of phospho(S10)p27Kip1 precedes the CDKI accumulation and increases its half-life. Finally, ATRA-treated nuclear LAN-5 extracts showed an enhanced capability of phosphorylating p27Kip1 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 p27Kip1 on S10. In turn, this event causes the stabilization of p27Kip1 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 precursors cells 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 p27Kip1 (8–12).

p27Kip1 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. p27Kip1 cellular content is regulated by intricate post-translation mechanisms and proteasome degradation (16–18). Several pieces of evidence suggest that p27Kip1 degradation is controlled by two distinct ubiquitination processes that occur in the nuclear and cytosolic compartments. Nuclear ubiquitin-dependent p27Kip1 degradation requires a previous phosphorylation on T187 catalyzed by active CDK2 in late G1 and S phases (19–22). T187 phosphorylation permits p27Kip1 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 points to the CDKI nuclear localization as a pivotal mechanism for controlling p27Kip1 level and function. Nuclear degradation requires the import of the protein, which has been proposed to involve the interaction of p27Kip1 with the nuclear pore protein Nup50/NPAP60 (32). Phosphorylation on T157 or T198, due to activated AKT/protein kinase B, hampers nuclear import (33, 34) and decreases p27Kip1 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 p27Kip1 binding to the carrier protein CRM1 (35, 36). Additional experiments suggest that Jun activation domain-binding protein 1 (Jab1) participates to the nuclear export of p27Kip1 by functioning as an assembly factor (37). However, recently, the phosphorylation on S10 as a pivotal step for nuclear export of p27Kip1 has been questioned seriously (38), and the postsynthetic modification seems to increase solely the CDKI stability (39).

The kinase (or kinases) catalyzing p27Kip1 phosphorylation on S10 has not been definitely identified (35, 36, 40, 41). Two enzymes...
have been proposed to catalyze the reaction. Human kinase interacting stathmin (hKIS) has been reported to phosphorylate p27^Kip1 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/dyrk1B 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 p27^Kip1 in G0 (38) and showed that this modification occurs only during G1-G0 transition. In conclusion, although the majority of phosphorylated p27^Kip1 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 p27^Kip1 up-regulation in the ATRA activity, we decided to investigate the molecular mechanism(s) by which retinoic acid induces p27^Kip1 increase. The obtained results point to p27^Kip1 phosphorylation on S10 as a major target of ATRA action.

Materials and Methods

Materials. ATRA was from Sigma Chemical Co. (St. Louis, MO), AM-S80, methoprene 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 BIMOL 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 p27^Kip1 coding sequence (plasmid: catalytic subunit) and λ protein phosphatase were obtained from Cell Signaling Technology (Beverly, MA). The mouse p27^Kip1 coding sequence cloned into the pcDNA3 plasmid (i.e., Mirk/dyrk1B 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 p27^Kip1 in G0 (38) and showed that this modification occurs only during G1-G0 transition. In conclusion, although the majority of phosphorylated p27^Kip1 is modified on S10, the precise function of the phosphorylation and the kinase(s) catalyzing the reaction has not been identified definitely.

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Western blot analysis, immunoprecipitation, and protein phosphatase assays. Cell extracts were prepared as described by Borriello et al. (10). Immunoprecipitation and immunoblotting were done exactly as with Borriello et al. (10). The serine/threonine phosphatase activity was measured using the nonradioactive Serine/Threonine Phosphatase Assay System purchased from Promega.

Two-dimensional electrophoresis analyses. For two-dimensional gel electrophoresis separations, trichloroacetic acid–precipitated whole-cell extracts (250–400 μg proteins) or immunoprecipitated proteins were resuspended in a rehydration solution containing 8 mol/L urea, 0.5% CHAPS, 0.2% DTT, 0.5% IGP buffer, and 0.002% bromphenol blue and separated on Immobile linear pH 3-10 or 4-7 DryStrip gels by isoelectric focusing using the Ettan IPGphor system (Amersham Biosciences, Uppsala, Sweden). Isoelectric focusing was done for a total of 32,000 V, starting at 50 V and gradually raising the voltage to 8,000 V. The strips were then equilibrated for 15 minutes in 50 mmol/L Tris (pH 8.5), 6 mol/L urea, 30% glycerol, 2% SDS, 1% DTT, and 0.002% bromphenol blue before loading onto SDS-polyacrylamide gels (15% acrylamide) for separation according to molecular mass. After transfer on nitrocellulose membranes, p27^Kip1 was immunodetected as reported above.

The phosphatase treatments were done on immunoprecipitated p27^Kip1. Briefly, 400 μg cell lysate proteins were immunoprecipitated using anti-p27^Kip1 polyclonal antibodies. The immunoprecipitate was divided in two aliquots. One aliquot was resuspended in 50 μL phosphatase buffer [50 mmol/L Tris-HCl, 0.1 mmol/L Na2 EDTA, 5 mmol/L DTT, 0.01% laurel ether (Brij-35), 2 mmol/L MnCl2]. Then, λ protein phosphatase (400 units) was 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 p27^Kip1 (60 ng) was immunoprecipitated by anti-p27^Kip1 polyclonal antibodies immobilized onto protein A-agarose. The immunocomplexes were then resuspended in PKA kinase buffer and incubated with recombinant PKA (6 units) and [γ-32P]ATP (6 μCi). After the reaction, labeled p27^Kip1 was eluted from the immunocomplexes by using 100 mmol/L glycine-HCl and added to p27^Kip1 immunoprecipitated from cell extract. Finally, the mixture was separated by two-dimensional PAGE and blotted on a nitrocellulose membrane. The blot was finally analyzed by autoradiography and then subjected to Western blotting.

Results

ATRA induces a Skp2-independent p27^Kip1 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 p27^Kip1 (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 p27^Kip1 increase are observable after 12 to 14 hours and precede the growth arrest, which becomes evident only after 24 to 48 hours of treatment.

The increase of p27^Kip1 level has been correlated to its degradation impairment. Indeed, we and others (10–12) showed that cell extracts, prepared from 24- and 48-hour ATRA-treated cells, degrade p27^Kip1 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 p27^Kip1 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 p27^Kip1, 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 CDKI 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 p27Kip1 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 p27Kip1 isoforms. Several mechanisms might explain the increase of p27Kip1 in the nuclear compartment. Recently, it has been described in prostate cells that p27Kip1 nuclear increase is due
Mechanisms of Retinoic-Dependent p27kip1 Increase

Figure 3. Two-dimensional analysis of p27kip1 in LAN-5 cells. A, Western blot analysis of the two-dimensional gel electrophoresis of p27kip1 immunoprecipitated from LAN-5 cells. Bottom, different p27kip1 isoforms were numbered according to their position relative to a computer scale of isoelectric point shifts generated by the addition to recombinant human p27kip1 of entire numbers of negative charges (see Methods). B, Western blot analysis of the two-dimensional gel electrophoresis pattern of p27kip1 from ATRA-treated LAN-5 cells before (Con) and after the treatment with protein phosphatase (+PPase). C, human recombinant p27kip1 were 32P labeled in vitro with recombinant PKA. The protein was added to p27kip1 immunoprecipitated from ATRA-treated LAN-5 cell extract. Finally, the mixture was separated by two-dimensional gel electrophoresis and then blotted onto a nitrocellulose paper. The paper was analyzed either for the labeling of p27kip1 (top) or for the labeling of p27kip1 (P32p27, bottom). D, LAN-5 cells were grown with or without 5 μmol/L ATRA and collected after 4 and 8 hours. Then, nuclear and cytosolic fractions were prepared as in Materials and Methods and analyzed by two-dimensional PAGE/immunoblotting. The various isoforms of the protein were evidenced as in (A).

An additional mechanism might be the impairment of p27kip1 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, p27kip1 was immunoprecipitated, and its isoforms were resolved by two-dimensional electrophoresis (focusing range, pH 4-7 linear gradient) and then detected by immunoblottting. Under these conditions, multiple forms of p27kip1 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 p27kip1 (form 0) focused at pH ~6.5, which is close to the calculated isoelectric point of human p27kip1 (pH 6.54; as established by using the Compute pI/Mw tool provided by the ExPASy Web site).

Besides p27kip1 form 0, all the other forms (except form 1) focused at positions that roughly correspond to that 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 (HPO42-; ref. 44), p27kip1 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 p27kip1 protein (data not shown). Second, the hypothesis that spots 2, 3, and 4 were phosphosioforms was shown by their disappearance after protein phosphatase treatment (Fig. 3B). Importantly, the treatment did not cause the loss of form 1. Third, recombinant p27kip1 was in vitro phosphorylated with recombinant activated PKA, and the labeled CDKI was mixed with p27kip1 immunoprecipitated from cell extract. As shown in Fig. 3C, only spots 2, 3, and 4 comigrated with the labeled phosphorylated p27kip1 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 produced 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.

p27kip1 phosphorylation on S10 is activated by ATRA. The ATRA-dependent changes of p27kip1 phosphorylation status prompted us to identify the phosphorylated residue(s) of the nuclear and cytosol CDKI. p27kip1 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 p27kip1 is Ser10 (38, 39), we tested the capability of several antisera (either commercial or kindly given by several investigators) to specifically recognized p27kip1 isofom phosphorylated on Ser10. 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 p27kip1 (Fig. 4A). Then, we prepared WT and S10A mutant p27kip1 and used the proteins as substrates of activated PKA. As shown in Fig. 4B, the antiserum clearly reacted with in vitro phosphorylated WT p27kip1, whereas it did not recognize phosphorylated S10A mutant p27kip1. Finally, when tested in two-dimensional PAGE/immunoblotting, the various isoforms of the protein were evidenced as in (A).
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)p27Kip1 after 4 hours (Fig. 4B), a time interval that clearly precedes the increase of nuclear p27Kip1.

To corroborate our observations using other experimental models, we searched for neuronal-like cells that respond to ATRA with a concomitant increase of p27Kip1. We found that PC12, a rat pheochromocytoma cell line, treated with ATRA, showed a rapid nuclear up-regulation of p27Kip1 (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)p27Kip1 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 p27Kip1 half-life (t1/2). An identical result was obtained by evaluating the nuclear phospho(S10)p27Kip1 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)p27Kip1 after ATRA addition: (a) the inhibition of a protein phosphatase that dephosphorylates the p27Kip1 phosphoisoform and (b) the activation of a specific p27Kip1 kinase.

To evaluate the importance of protein phosphatase activities, we incubated LAN-5 cells with okadaic acid or calyculin A, two powerful inhibitors of protein phosphatase activities, and evaluated the level of p27Kip1. The compounds neither increased p27Kip1 level nor changed its cellular distribution (Fig. 5E). Then, we investigated the effect of ATRA on the activity of protein phosphatase 2 (PP2) isomorphs (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)p27Kip1 is not correlated to the modulation of protein phosphatase activity.

Effect of ATRA on p27Kip1 kinase activity. To further unravel the ATRA effect on p27Kip1 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)p27Kip1.

Because we were unable to identify the nuclear enzyme that phosphorylated the CDKI on Ser10, we decided to use the antisera against phospho(S10)p27Kip1 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 isoform. As shown in Fig. 6A, the addition of nuclear extract strongly increased the signal of phospho(S10)p27Kip1, 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 p27Kip1 phosphorylation profiles. Particularly, in the nucleus, the accumulation of the monophosphorylated form is observable, whereas in the cytosol the unmodified p27Kip1 increases. Importantly, these changes are evident well before changes in total p27Kip1 content, indicating that they precede the accumulation of the protein. Thus, the up-regulation of nuclear p27Kip1 phosphorylation represents a precocious event in ATRA effect on cell cycle.

Then, we identified by immunologic approaches the increased CDKI phosphoisoform as p27Kip1 phosphorylated on S10. We also showed that the accumulation of nuclear phospho(S10)p27Kip1 is associated with the CDKI 1/2 elongation as also observed by others (38, 39). Thus, we speculated that ATRA increases nuclear p27Kip1 by up-regulating the amount of phospho(S10)p27Kip1.

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 p27Kip1, thus making strongly questionable the obtained conclusions. Conversely, we decided to investigate by direct biochemical approaches the correlation between ATRA and p27Kip1 phosphorylation.

A possible explanation for the increase of the phosphorylated p27Kip1 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 suggest 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 p27Kip1 kinases have been putatively identified [i.e., AKT (33, 34), ERK1/2 (51), hKIS (40), and Mirk/dyrk1B (41)]. AKT phosphorylates p27Kip1 on threonine residues (T157 or T198), making unlikely that the enzyme is responsible for p27Kip1 serine phosphorylation. However, we did experiments that definitely excluded AKT as the enzyme responsible for p27Kip1 phosphorylation on Ser10.

Up-regulation of ERK1/2 activity causes a p27Kip1 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 resulted in the increase of nuclear p27Kip1. 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 p27Kip1 up-regulation.

Boehm et al. (40) reported that hKIS phosphorylates p27Kip1 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 p27Kip1 and the inhibition of cell growth. This finding putatively excludes hKIS as the ATRA-dependent kinase, which phosphorylates p27Kip1 (52). 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 p27Kip1 on S10 and increases during the 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)p27Kip1 is ubiquitous (39, 41). Second, the enzyme is strongly active in G0 (41), whereas
in this phase, a scarce phosphorylation of p27Kip1 has been recently shown (38). Because we ruled out the known p27Kip1 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 phospho(S10) p27Kip1. By this method, we evidenced that ATRA increases the p27Kip1 kinase activity in the nuclear extracts. Such a finding might explain the data obtained by the two-dimensional analysis and the increase of p27Kip1 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 PKA as shown by the lack of effect of specific inhibitors. Studies in development are to further characterize the p27Kip1 kinase activity.

In conclusion, our study shows for the first time that (a) p27Kip1 increases only after 8 hours of ATRA treatment; (b) the upregulation occurs only in the nuclear compartment, whereas the cytosolic content remains unmodified up to 24 hours; and (c) the early CDKI up-regulation is independent on the decrease of Skp2 level and the consequent impairment of p27Kip1 degradation system. The CDKI accumulation seems to be due to stabilization of nuclear p27Kip1 and is associated to the increase of kinase activity able to phosphorylate the protein on S10.

Finally, our study on p27Kip1 isoforms indicates the occurrence on the protein of several phosphorylation sites (at least three), which might be contemporaneously modified, and a new posttranslational 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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