Retinoid-induced G1 Arrest and Differentiation Activation Are Associated with a Switch to Cyclin-dependent Kinase-activating Kinase Hypophosphorylation of Retinoic Acid Receptor α

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Cell cycle G1 exit is a critical stage where cells commonly commit to proliferate or to differentiate, but the biochemical events that regulate the proliferation/differentiation (P/D) transition at G1 exit are presently unclear. We previously showed that MAT1 (ménage à trois 1), an assembly factor and targeting subunit of the cyclin-dependent kinase (CDK)-activating kinase (CAK), modulates CAK activities to regulate G1 exit. Here we find that the retinoid-induced G1 arrest and differentiation activation of cultured human leukemic cells are associated with a switch to CAK hypophosphorylation of retinoic acid receptor α (RARα) from CAK hyperphosphorylation of RARα. The switch to CAK hypophosphorylation of RARα is accompanied by decreased MAT1 expression and MAT1 fragmentation that occurs in the differentiating cells through the all-trans-retinoic acid (ATRA)-mediated proteasome degradation pathway. Because HL60R cells that harbor a truncated ligand-dependent AF-2 domain of RARα do not demonstrate any changes in MAT1 levels or CAK phosphorylation of RARα following ATRA stimuli, these biochemical changes appear to be mediated directly through RARα. These studies indicate that significant changes in MAT1 levels and CAK activities on RARα phosphorylation accompany the RARα-induced G1 arrest and differentiation activation, which provide new insights to explore the inversely coordinated P/D transition at G1 exit.

The cyclin-dependent kinase (CDK)1-activating kinase (CAK), a trimeric CDK7-cyclin H-MAT1 (ménage à trois 1) complex, was originally implicated in cell cycle control by its ability to phosphorylate and activate CDKs (1, 2). Previous studies demonstrated that CAK regulates cell cycle G1 exit both by phosphorylation activation of cyclin D-CDK complexes (3–7) and by phosphorylation inactivation of retinoblastoma tumor suppressor protein (pRb) (8). CAK is a subcomplex of transcription factor IIIH (TFIIH) (9–12) and a kinase of TFIIH that phosphorylates the COOH-terminal domain of the smallest subunit of RNA polymerase II for transcription initiation (9, 13–15). Thus, CAK is considered a cross-road regulator in linking cell cycle control with transcription. Recently, distinct regions of MAT1 have been shown to regulate CAK kinase and TFIIH transcription activities (16). To date, comprehensive studies demonstrate that MAT1 regulates CAK substrate specificity and protein-protein interactions, i.e. MAT1 mediates the association of CAK with core TFIIH and shifts CAK substrate preference from CDK2 to the COOH-terminal domain (12, 14, 17, 18). Mice lacking MAT1 are unable to enter S phase and are defective in RNA polymerase II phosphorylation (19). Antibody abrogation of MAT1 induces cell cycle G1 arrest (20); and MAT1 regulates the interaction and phosphorylation of CAK with tumor suppressor p53 (21), octamer transcription factors (22), pRb (8), and retinoic acid receptor α (RARα) (23).

Among the above substrates of CAK, RARα is involved mainly in differentiation regulation. RARα belongs to the superfamily of nuclear ligand-activated transcriptional regulators, the retinoic acid receptors. RARα is a phosphoprotein (23–26) and mediates the action of retinoids in myeloid differentiation (27–29). In HL60 leukemic cells, the all-trans retinoic acid (ATRA)-induced differentiation is mediated directly through the RARα (30, 31). Indeed, a subclone of HL60 (designated HL60R) harbors a truncated AF-2 domain of RARα (RARαΔAF-2) and is resistant to differentiation induction by ATRA. However, introducing a normal RARα cDNA into these cells restores their differentiating response to ATRA (30–32). Both the ligand-dependent transcriptional activation function AF-2 (located in the RARα E region) and the ligand-independent transcriptional activation function AF-1 (located in the RARα A/B region) are involved in cell differentiation (33, 34). In vitro studies show that CAK phosphorylates RARα at both the A/B and F regions (23). However, the precise molecular mechanisms whereby CAK phosphorylates RARα and its functional consequences remain unknown.

The decision of cells to differentiate is commonly made in cell cycle G1 phase, and differentiation induction requires cell cycle arrest (35–38), but little is known about how the cell cycle machinery coordinates cell cycle arrest with differentiation activation. Given that CAK regulates cell cycle G1 exit for S-phase entry (3–8, 20) and that RARα, a key player in myeloid differentiation (36, 39, 40), is a substrate for CAK in Cos-1 cells...
(23), we investigated whether there was any correlation between CAK-RARα signaling and ATRA-induced differentiation in cultured human leukemia cells. We found that ATRA-induced MAT1 reduction and CAK hypophosphorylation of RARα are RARα-dependent and are associated with G1 arrest and differentiation activation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human leukemia cells AML1 and Nalm6 (provided by Dr. Kohn), NB4 (provided by Dr. Lanotte), HL60, HL60R, REH, Jurkat, lymphoma U937, and human osteosarcoma U2OS cells were cultured in RPMI 1640 plus 10% fetal bovine serum. Human diploid fibroblast IMR90 and human transformed embryonal kidney 293 cells were cultured in minimal essential medium (Eagle) supplemented with 10% fetal bovine serum. Human Ewing's sarcoma TC71, TC32, human rhabdomyosarcoma (RD), and human Ewing's spfNET CH100L cells are USC pathology cell lines and were cultured in RPMI 1640 containing 10% fetal bovine serum. Cell lines were purchased from ATCC unless otherwise specified. ATRA and protease inhibitor MG-132 were from Sigma. Either 1 or 5 μM of ATRA were used to treat the cells, and similar effects were observed between the two concentrations.

**In Vivo Phosphorylation and Immunoprecipitation**—Immediately before labeling, subconfluent cells (5 × 10^6/ml) were adjusted to 1 × 10^7/ml and cultured in phosphate-free complete medium for 1 h. Then, 1 × 10^6/ml cells were incubated with 125 μCi of [32P]orthophosphate (ICN) in serum-free medium for 2 h at 37°C. Cells were centrifuged and harvested in ice-cold phosphate-buffered saline. Nuclear protein extraction was performed at 4°C using a modified high salt extraction buffer (41). The same amounts of nuclear proteins from each sample were used for immunoprecipitation as described (8). The resulting immunoprecipitates were resolved by SDS-PAGE, electrotransferred onto polyvinylidene difluoride membrane, and autoradiographed. All anti-human polyclonal and monoclonal antibodies used in immunoprecipitation were purchased from Santa Cruz Biotechnology.

**Western Blotting and Cell Proliferation Analyses**—Western blotting was performed as described previously (8). All anti-human polyclonal and monoclonal antibodies were purchased from Santa Cruz Biotechnology. The rate of cell duplication determined by cell counting was described before (20).

**Analysis of Cell Cycle Profile and Detection of Cytdifferentiation Antigen**—Cell cycle profile was analyzed as described before (20). A direct immunofluorescence staining technique was applied to analyze myeloid differentiation marker CD11b. Cells were exposed to phosphatidylinositol-conjugated CD11b antibodies at 4°C for 30 min and fixed with fresh 1% paraformaldehyde. The antigens were then determined by a FACSScan flow cytometer (BD Biosciences). The percentage of positive cells and the mean associated fluorescence were quantitated using a FACSscan analyzer (CellQuest software V3.2). Control studies were performed with fluorescein isothiocyanate-conjugated and phosphatidylinositol-conjugated antihuman CD45, and fluorescein isothiocyanate-conjugated and phosphatidylinositol-conjugated non-binding mouse IgG1 (IgG1). All antibodies and mouse IgG were purchased from BD Biosciences.

**Characterization of Nuclear Segmentation**—Subconfluent cells with or without ATRA treatment were fixed by methanol and stained with Wright-Giemsa (Sigma). The mature nuclear segmentation of leukemic cells was evaluated under a Zeiss Axioplan microscope. Images were color balanced in Adobe Photoshop.

**RESULTS**

**CAK Hypophosphorylation of RARα Accompanies the ATRA-induced G1 Arrest and Differentiation Activation**—Since CAK interacts with and phosphorylates RARα in vitro (20), we wanted to determine whether the ATRA-induced terminal differentiation of HL60 cells was associated with any changes of CAK-RARα signaling. HL60 cells following different periods of ATRA and ATRA were in vivo labeled with [32P]orthophosphate. We used CDK7 antibody to immunoprecipitate the CAK-bound and RARα from the labeled cells for visualizing CAK-RARα signaling by autoradiography. As we expected, CAK interacted with and phosphorylated RARα in vivo because CDK7 antibody brought down phosphorylated RARα and phosphorylated CDK7 simultaneously (Fig. 1A). We found that CAK hypophosphorylated RARα in proliferating cells. However, CAK hyperphosphorylated RARα was inhibited about 40% after 24 h of ATRA stimuli and then diminished to over 90% after 96 h of ATRA stimuli (densitometer results not shown) (Fig. 1A). CAK activity as represented by CDK7 autophosphorylation also decreased significantly, showing a correspondence to decreased RARα phosphorylation (Fig. 1A). Western analyses of CAK subunits and their associated RARα were performed by using this same blot. The results showed that RARα and CDK7 antibodies recognized RARα and CDK7, respectively, at the corresponding molecular weight positions of their phosphorylations (Fig. 1A). CDK7 polyclonal antibodies distinguished both forms of the autophosphorylated CDK7 (P-K7) and the autohypophosphorylated CDK7 (K7). Following ATRA stimulation, the P-K7 diminished gradually, whereas the levels of K7 correspondingly increased (Fig. 1A). The dynamic levels of P-K7 and K7 corresponded well with the dynamic status of RARα hyperphosphorylation and RARα hypophosphorylation (Fig. 1A). Hence, these results show that ATRA induces a switch to CAK hypophosphorylation of RARα in differentiating cells from CAK hyperphosphorylation of RARα in proliferating cells and that this reduced CAK phosphorylation of RARα is associated with a decreased CAK activity.

Surprisingly, we observed a 30-kDa MAT1-associated fragment (M30) that was immunoprecipitated by CDK7 antibodies and recognized by MAT1 polyclonal antibodies in Western blotting (Fig. 1A). This indicated that M30, together with MAT1, was within the CAK complex. We found that gradual diminution of M30 paralleled the developments of both RARα hypophosphorylation and CDK7 hypophosphorylation, whereas the levels of both cyclin H and RARα appear unrelated to this dynamic phosphorylation pattern (Fig. 1A). The results suggest that ATRA-induced diminution of M30 within the CAK complex might be associated with the dynamic changes of CAK phosphorylation of RARα.

We further monitored, in parallel, the relationship between CAK-RARα signaling and P/D transition. We found that the reduction in RARα phosphorylation was associated with the occurrence of pRb hypophosphorylation and G1 arrest after 48 h of ATRA stimuli (Fig. 1, A–C). Proliferation was halted after 72 h of either 1 or 5 μM of ATRA treatment, and then cell numbers remained stable (Fig. 1D). During this period, differentiation proceeded as shown by CD11b expression and nuclear segmentation (Fig. 1, E and F). Interestingly, although we found pRb hypophosphorylation under ATRA stimuli (Fig. 1B), there was no change in either cyclin D1 expression or CDK4 phosphorylation by Western analyses (data not shown). Hence, these results indicate that ATRA concurrently induces CAK hypophosphorylation of RARα and cyclin D/CDK4-independent pRb hypophosphorylation. The dynamic switch to CAK hypophosphorylation of RARα was associated with the transition from actively proliferating cells to G1 arrest that accompanies the terminal myeloid differentiation.

**MAT1 Expression and the Origin of M30**—The aforementioned results show that MAT1-associated M30 exists within CAK complex and that ATRA-induced diminution of M30 is associated with reduced CAK phosphorylation of RARα. Therefore, we explored the following: (a) the relationship between MAT1 and M30; and (b) the origin of M30. By Western analyses of leukemic HL60 and NB4 cells, we found that M30 always was associated with MAT1 in proliferating cells. Total cellular MAT1 expression was inhibited about 50% after 48 h of ATRA stimuli (Fig. 2, A and B). M30 was decreased significantly more, approaching 90–100% reduction after 48–72 h of ATRA stimuli (Fig. 2, A and B), which corresponded to the reduced levels of M30 within the CAK complex (Fig. 1A). In contrast to the reduction of MAT1/M30 by ATRA, the total CDK7 protein...
level remained unchanged (Fig. 2, A and B, densitometer results not shown). To investigate whether M30 is associated with MAT1 in other tumor cells, we analyzed several solid tumor and leukemic cell lines using Western blotting. We found that MAT1 antibodies recognized several fragments ranging from about 20 to 30 kDa in certain tumor cell lines. Further, not only was M30 consistently present but also MAT1 expression was enhanced in these tumor cells compared with normal IMR90 cells (Fig. 2, C and D). Because ATRA-induced diminution of MAT1/M30 is associated with G1 arrest and differentiation activation in differentiating cells (Figs. 1 and 2, A and B), these results suggest that the overexpressed MAT1 and the high levels of M30 in these tumor cells may be related to uncontrolled cell proliferation.

We anticipated that M30 could be one of the following: (a) a degraded MAT1 fragment; (b) a splicing variant of the MAT1 gene; or (c) a new gene of sequence homology with MAT1. To address these issues we used multiple pairs of specific and

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**Fig. 1. In vivo CAK hypophosphorylation of RARα in HL60 cells correlates with P/D transition.** A, in vivo phosphorylated 32P-labeled RARα and CDK7 were immunoprecipitated with CDK7 antibodies. Further, this same blot was used for Western analyses of CDK7, MAT1, RARα, and cyclin H. A MAT1-associated M30 fragment was recognized by MAT1 polyclonal antibodies. P-K7, autohyperphosphorylated form of CDK7; K7, autohypophosphorylated form of CDK7; WB, Western blot; IP, immunoprecipitate. B, Western detection of in vivo phosphorylation status of pRb. P-pRb, hyperphosphorylated form of pRb; pRb, hypophosphorylated form of pRb. C, progressively developed G1 arrest under ATRA stimuli. D, cell proliferation under ATRA stimuli. The growth curves represent the mean ± S.D. from the cells of triplicate wells. E, analysis of differentiation antigen CD11b. Samples I–IV minus ATRA were stained with mouse IgG, CD45, and CD11b antibodies as indicated for controls. Sample V was stimulated with ATRA for 120 h and then stained with CD11b antibodies. 40% of cells were positive of CD11b expression in sample V. F, nuclear segmentation was markedly evident after 120 h of ATRA stimuli.
ATRA Induces Proteasome-dependent Degradation of MAT1

Previous studies have demonstrated that ATRA induces a proteasome-dependent degradation of retinoic acid receptors (42, 43). Thus, we wanted to determine whether ATRA-induced reduction of MAT1/M30 was similarly related to enhanced protein degradation. To test this, HL60 cells were incubated with or without ATRA for 48 h, and then cells were exposed either to vehicle or to protease inhibitor MG-132 for an additional 24 h before harvest. Since ATRA stimuli lead to RAR degradation but have no effect on the levels of CDK7 and cyclin H, we used RARs as a positive control, whereas CDK7 and cyclin H were used as negative controls in parallel. We found that the levels of MAT1, M30, and RARs decreased with ATRA stimuli (Fig. 3A, lanes 2) but then could be overcome by the addition of MG-132 (Fig. 3A, lanes 3). In contrast, neither CDK7 nor cyclin H was affected by ATRA stimuli or MG-132 treatment (Fig. 3B, lanes 2 and 3). In an extension of this approach, we blocked the proteasome pathway first by treating HL60 cells with MG-132 for 8 h. We then added ATRA for an additional incubation of 60 h. Compared with the cells with ATRA stimuli alone (Fig. 3C, lane 2), the cells with pretreatment of proteasome inhibitor blocked ATRA-induced MAT1/M30 degradation (Fig. 3C, lane 3). These results demonstrate that ATRA inhibits MAT1 expression and M30 formation via an ATRA-induced proteasome pathway. Since CAK activity is MAT1 dose-dependent (8, 18, 22, 44) and the reduction of MAT1/M30 is associated with decreased CAK phosphorylation of RARα (Fig. 1A), these results suggest that ATRA-induced diminution of MAT1/M30 via the proteasome pathway may inhibit CAK activities on RARα phosphorylation.

RARα Activation by ATRA Is Required for Both the Reduction of MAT1/M30 and the Switch to CAK Hypophosphorylation of RARα—ATRA, signaling via the ligand-dependent AF-2 domain of RARα in HL60 cells containing a wild type RARα (RARαWT) (30–32), inhibits MAT1/M30 through a proteasome degradation pathway (Fig. 3) and induces CAK hypophosphorylation of RARαs in ATRA-induced P/D transition (Fig. 1). Thus, we utilized HL60R cells, which harbor RARαΔAF-2 and are resistant to differentiation by ATRA (30–32), to explore the relationship of ATRA-induced P/D transition with RARαs activation, the dynamic changes of MAT1/M30 levels, and the CAK activities on RARα phosphorylation. We first tested whether MAT1 expression and M30 formation would be inhibited by ATRA-activated proteasome pathway in HL60R cells. We treated HL60 and HL60R cells with ATRA for 48 h and then added MG-132 for an additional incubation of 24 h before harvest. Western analyses showed that in contrast to HL60 cells showing a reduction of MAT1/M30 by ATRA (Fig. 4A, lane

**Fig. 2. MAT1 expression and the origin of M30.** A and B, Western analyses of MAT1 expression and M30 formation following ATRA stimuli of HL60 and NB4 cells. Actin was used as a loading control. C and D, Western analysis of MAT1/M30 in tumor cells and normal IMR90 cells. E, M30 is likely a COOH-terminal deleted MAT1 fragment. Lanes labeled 1 were blotted with full-length anti-MAT1 antibodies, lanes labeled 2 with NH2-termini anti-MAT1 (against about 1–30 amino acids), and lanes labeled 3 with C termini anti-MAT1 (against approximately 280–309 amino acids).
Fig. 3. ATRA inhibits M30 and MAT1 via an ATRA-activated protease pathway. A, Western analyses showed that MG-132 overcomes the ATRA-induced degradation of MAT1/M30 and RARα. Lanes labeled 1 were treated with vehicles only. Lanes labeled 2 were treated with ATRA (1 μM) for 72 h. Lanes labeled 3 were treated with ATRA for 48 h first, and then 0.2 μM MG-132 was added for an additional incubation of 24 h. MG-132 was dissolved in MeSO (DMSO) and ATRA in ethanol. B, neither ATRA nor MG-132 affects the levels of CDK7 and cyclin H. The sample order is the same as described in the A section. C, Western analyses showed that MG-132 prevents the ATRA-induced degradation of MAT1/M30. Lane 1 was with vehicles only. Lane 2 was treated with ATRA for 68 h. Lane 3 was treated with MG-132 (0.5 μM) first for 8 h and then added ATRA for an additional incubation of 60 h.

2) but an overcoming by MG-132 (Fig. 4A, lane 3), there was virtually no change of MAT1/M30 in HL60R cells (Fig. 4A, lanes 5 and 6). Second, we compared MAT1 expression and M30 formation in HL60 and HL60R cells by Western analyses. In contrast to HL60 cells, HL60R cells retained high levels of MAT1/M30 under ATRA stimuli (Fig. 4B). These results show that RARα activation by ATRA is required for inhibition of MAT1 expression and M30 formation.

Next, we examined the relationship between the levels of MAT1/M30, CAK phosphorylation of RARα, and G1 arrest/differentiation activation in HL60R cells. Similarly in studies of CAK-RARα signaling as performed in HL60 cells (Fig. 1), we labeled HL60R cells in vivo with [32P]orthophosphate and immunoprecipitated CAK-bound RARα using CDK7 antibodies. We found no change in either CDK7 autophosphorylation or CAK phosphorylation of RARα following ATRA stimuli of the HL60R cells (Fig. 4C). Further, Western analyses of this same blot showed that high levels of MAT1/M30 were retained in the CAK complexes and corresponded well to both CDK7 autohyperphosphorylation and RARα hyperphosphorylation (Fig. 4C). Also, as we expected that whereas CAK hyperphosphorylation of RARα was retained in HL60R cells, the cells remained virtually continuous cycling (Fig. 4D) and proliferating (Fig. 4E) without detectable CD11b expression (Fig. 4F) or morphology change (Fig. 4G) following ATRA stimuli. Thus, these results indicate that ATRA-induced RARα activation via the AF-2 domain of RARα is critical to the changes in MAT1/M30 levels and CAK activities on RARα phosphorylation that occur in the differentiating HL60 cells (Fig. 1). Moreover, in the ATRA-treated HL60R cells the lack of changes in MAT1/M30 levels or CAK activities on RARα phosphorylation is correlated with the absence of both G1 arrest and differentiation activation (Fig. 4). Hence, these results strengthen the observed associations between MAT1/M30 levels, CAK phosphorylation of RARα, and terminal myeloid differentiation.

CAK Phosphorylation Regulation of RARα Is Independent of RARα Degradation—Because ATRA induces RARα degradation (42, 43) (Fig. 3A), we investigated whether the reduction in CAK phosphorylation of RARα was related to the diminished levels of RARα substrate resulting from RARα degradation. Using Western analyses, we first monitored ATRA-induced RARα degradation in HL60 cells from 1 h to 8 days. We found that the onset of RARα degradation was evident after 7 h of ATRA stimuli, but afterward the RARα levels remained relatively stable for up to 8 days (Fig. 5A). RARα also maintained a relatively steady level within the CAK complex after its onset of degradation (Fig. 1A). In contrast to this pattern of RARα degradation, the reduction in CAK phosphorylation of RARα was not observed until 24 h after ATRA stimuli; it then diminished about 70% to more than 90% after 48–96 h of ATRA stimuli (Figs. 5B and 1A). These marked differences in the kinetics of degradation versus the pattern of CAK phosphorylation of RARα suggest that CAK phosphorylation regulation of RARα under ATRA stimuli is independent of RARα degradation.

DISCUSSION

In a variety of different tissues, P/D transition is a process of coordinating cell cycle arrest with differentiation activation. However, the physiological phenomena and the regulatory mechanisms that are involved in coordinating these inverse molecular events remain unclear. We present evidence to show an ATRA-induced transition from proliferating to differentiating cells, where the ATRA-mediated MAT1 reduction and CAK hypophosphorylation of RARα may be involved in coordination of G1 arrest and differentiation activation of these cultured leukemic cells.

CAK-RARα Signaling May Be Instrumental in Coordinating G1 Arrest and Differentiation Activation—CAK regulates cell cycle G1 exit for S-phase entry (3–8, 20), and RARα is involved in regulating the terminal differentiation of different cell types (27–29, 36, 39, 40). The fact that CAK interacts with and phosphorylates RARα in vitro (23) led us to determine whether there was any in vivo association between CAK phosphorylation of RARα and ATRA-induced differentiation of HL60 cells. Since RARα is also phosphorylated by protein kinase A and a
variety of proline-directed protein kinases (23, 26, 45), immunoprecipitation of in vivo phosphorylated RARα by RARα antibodies cannot distinguish the signaling specificity of RARα by CAK. Thus, our experimental strategy was to immunoprecipitate in vivo CAK-bound RARα using CDK7 antibodies, which not only ensures the specificity of RARα phosphorylation by CAK but also allows us to visualize RARα phosphorylation status and CAK activities simultaneously. By analyzing these immunoprecipitates, we observe that CAK interacts with and phosphorylates RARα in vivo (Figs. 1A, 4C, and 5B). ATRA-induced G1 arrest and differentiation activation are associated with markedly decreased CAK activities and CAK phosphorylation of RARα (Fig. 1). Similar events in P/D transition also were observed in ATRA-sensitive NB4 cells (data not shown). In contrast, we did not observe any such reduced CAK phosphorylation of RARα as well as its association with G1 arrest and differentiation activation in ATRA-treated HL60R cells that harbor RARαΔAF-2 (Fig. 4, C-G). Hence, our data indicate that ATRA-induced diminution of CAK activities on RARα phosphorylation is mediated directly via the RARα and suggest that in HL60 cells containing RARαWT, ATRA-induced CAK hypophosphorylation of RARα may coordinate G1 arrest with differentiation activation.

MAT1-dependent CAK Activities—What are the factors that mediate the switch to CAK hypophosphorylation of RARα in ATRA-induced differentiation of leukemic cells? Previous stud-

![Diagram](image-url)
ies demonstrate that MAT1 determines CAK substrate specificity and further enhances CAK activities in either a dose-dependent manner or via MAT1-mediated protein-protein interactions (8, 14, 18, 19, 21–23, 44). MAT1 mRNA is overexpressed in multiple tumor cell lines (46), and differentiation induction by ATRA in NB4 cells is associated with reduction of MAT1 mRNA (47). Also, MAT1 protein is overexpressed in multiple solid tumor cell lines and leukemic cell lines (Fig. 2, C and D). These data therefore indicate that high levels of both MAT1 mRNA and MAT1 protein are associated with enhanced cell proliferation. Importantly, we consistently observe that a unique M30, likely derived from cleavage of MAT1, was produced along with MAT1 overexpression in tumor cell lines (Fig. 2, C and D). MAT1/M30 existing in proliferating cells but not in differentiating cells (Figs. 1 and 2, A and B) are immunoprecipitated together by CDK7 antibodies (Figs. 1A, 4C, and 5B). Thus, MAT1 may form a CAK complex together with the exceeded CAK complexes formed by overexpressed MAT1 to alter CAK substrate specificity on RARα phosphorylation in these leukemic cells. Indeed, these high levels of MAT1/M30 were consistently associated with CAK hyperphosphorylation of RARα in the actively proliferating HL60 cells, but these levels were markedly diminished by ATRA stimuli and thus were associated with the reduced CAK phosphorylation of RARα in the differentiating cells (Figs. 1, 2, A and B, and Fig. 5B). In HL60 cells containing RARα WT, MAT1 overexpression and in particular the M30 formation are markedly diminished by ATRA (Figs. 1A, 2A, 2B, and 5B) through a proteosome degradation pathway (Fig. 5). However, in clear contrast, there is no change of MAT1/M30 in the HL60R cells harboring RARαAF-2 following exposure to ATRA (Fig. 4, A–C). Thus, ATRA-activated RARα appears to modulate these dynamic changes of MAT1/M30 levels directly. These observations therefore suggest that the RARα-dependent MAT1 levels, and in particular the levels of the M30, might be important in regulation of CAK activities on RARα phosphorylation and that the ATRA-mediated proteosome degradation of MAT1/M30 may be a critical event in down-regulation of CAK-RARα signaling that is associated with $G_i$ arrest and differentiation activation of these leukemic cells.

Although ATRA-induced differentiation of leukemic cells is associated with degradation of RARα, we note that the pattern of RARα degradation either within or outside the CAK complex does not match the substrate stoichiometry of the gradually developing CAK hypophosphorylation of RARα (Figs. 1A and 5). In contrast, gradually inhibited MAT1/M30 by ATRA parallel gradually developed CAK hypophosphorylation of RARα (Figs. 1A and 5B). As CAK activities are known to be MAT1 dose-dependent (8, 18, 22, 44), these results therefore suggest that MAT1 reduction, rather than RARα degradation, may be a main factor to modulate CAK phosphorylation of RARα in ATRA-induced P/D transition.

The Significance of CAK Hypophosphorylation of RARα—What is the significance of the diminished CAK phosphorylation of RARα that accompanies the ATRA-induced P/D transition in HL60 cells? Such decreased RARα phosphorylation might reflect a generalized decrease in CAK activity because the ATRA-induced cells proceed from actively proliferating to terminally differentiating. Indeed, as discussed above, the markedly decreased MAT1/M30 levels via the ATRA-activated proteosome pathway may reduce CAK activities on RARα phosphorylation. Alternatively, the decreased RARα phosphorylation might be more directly involved in regulating the molecular events that accompany terminal myeloid differentiation. This latter hypothesis might appear counterintuitive, because a previous study in Cos-1 cells indicated that CDK7 phosphorylation of RARα was associated with enhanced RARα transcriptional activity (23), and thus reduced phosphorylation of RARα predicts reduced RARα activity. However, such reduced activity might indeed occur as a negative feedback mechanism during terminal myeloid differentiation. Alternatively, the functional significance of RARα phosphorylation might be markedly different in hematopoietic cells versus Cos-1 cells.

In summary, we find that ATRA-induced cell cycle $G_i$ arrest and differentiation activation of HL60 cells are associated with a markedly decreased CAK phosphorylation of RARα. An accompanying event is the ATRA-mediated degradation of MAT1/M30 via the proteosome pathway, which might play a critical role in modulating this decreased RARα phosphorylation by CAK. The potential role of MAT1/M30 in regulating CAK ac-
tivity on RARs phosphorylation as well as the functional significance of the switch to CAK hypophosphorylation of RARs in coordinating G1 arrest and differentiation activation are currently being explored in our laboratory. The detailed molecular and biochemical pathways regarding MAT1-mediated CAK-RARα signaling in control of the transition from actively proliferating to terminally differentiating cells might provide a mechanistic insight into new approaches for leukemia therapy.

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