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Published in:
Oncogene

DOI:
10.1038/sj.onc.1208090

2004

Oncogene (2004), 1–8

www.nature.com/onc

ORIGINAL PAPER

Regulation of the cyclin A1 protein is associated with its differential subcellular localization in hematopoietic and leukemic cells

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An important role of the cell cycle regulatory protein cyclin A1 in the development of acute myeloid leukemia (AML) was previously demonstrated in a transgenic mouse model. We have now turned our attention to study specific aspects of the activity and subcellular distribution of cyclin A1 using bone marrow samples from normal donors and patients with AML, as well as leukemic cell lines. We show that the localization of cyclin A1 in normal hematopoietic cells is nuclear, whereas in leukemic cells from AML patients and cell lines, it is predominantly cytoplasmic. In leukemic cell lines treated with all-*trans* retinoic acid (ATRA), cyclin A1 localized to the nucleus. Further, there was a direct interaction between cyclin A1 and cyclin-dependent kinase 1, as well as a major ATRA receptor, RARα, in ATRA-treated cells but not in untreated leukemic cells. Our results indicate that the altered intracellular distribution of cyclin A1 in leukemic cells correlates with the status of the leukemic phenotype.
Oncogene advance online publication, 18 October 2004; doi:10.1038/sj.onc.1208090

Keywords: cyclin A1; AML; subcellular localization; CDK1; RARα

Introduction

Hematopoietic stem cells undergo a decision either to self-renew and remain pluripotent or to differentiate into committed progenitor cells. These progenitor cells differentiate into precursor cells of different lineages in the bone marrow (Tenen, 2003). Disruption of the normal processes of cell proliferation, differentiation, and apoptosis during hematopoiesis may lead to leukemogenesis (Rabbitts, 1994; Look, 1997; Tenen, 2003). Some of the new therapeutic approaches in leukemia exert their effects by reprogramming the differentiation process that was altered in malignant cells. For example, all-*trans* retinoic acid (ATRA) can induce clinical remission in patients with acute promyelocytic leukemia (APL), a subtype of acute myeloid leukemia (AML) (He *et al*., 1999; Zhu *et al*., 1999). Certain leukemic cell lines respond to ATRA by switching from a state of uncontrolled proliferation, typical for malignant cells, to a state in which the cells undergo terminal differentiation like normal hematopoietic cells (Dimberg *et al*., 2002). It has been suggested that the role of ATRA in promoting myeloid differentiation in APL might be related to its ability to restore a normal subcellular localization of several leukemia-associated proteins such as PML and other nuclear domain-associated proteins (Koken *et al*., 1994; Weis *et al*., 1994, Faretta *et al*., 2001). RARα, a nuclear hormone receptor, mediates the response to ATRA by releasing corepressors and recruiting coactivators and leads to the induction of target genes for granulocytic differentiation (He *et al*., 1999).

Cell cycle control plays a fundamental role in cell differentiation and growth. The proper regulation of the cell cycle machinery, including cyclins, cyclin-dependent kinases (CDK), and the negative regulators CDK inhibitors (CDKI), is critical for hematopoiesis (Furukawa, 1997, 1998; Dao and Nolta, 1999). Several reports have also emphasized the importance of the dynamic subcellular localization of cell cycle regulatory molecules such as the CDKIs p27 and p21 for the differentiation of hematopoietic cells (Yaroslavskiy *et al*., 1999, 2001). Further, the dynamic subcellular localization of p27 was shown to result from the sequestration of cytoplasmic p27 by cyclin D3/CDK4 complexes (Reynisdottir and Massague, 1997).

Several lines of evidence have indicated a strong association between the aberrant expression of or mutations in cell cycle regulatory genes and leukemogenesis (Iida *et al*., 1997; Volm *et al*., 1997; Amanullah *et al*., 2000; Faderl *et al*., 2000). In particular, several studies have linked a novel A-type cyclin, cyclin A1, to leukemogenesis. Elevated levels of human cyclin A1 have been detected in myeloid hematological malignancies (Yang *et al*., 1997, 1999; Kramer *et al*., 1998). Importantly, targeted overexpression of cyclin A1 in

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Received 19 April 2004; revised 9 August 2004; accepted 13 August 2004

early myeloid cells initiated AML in transgenic mice (Liao *et al*., 2001), a functional study that demonstrated an important role of cyclin A1 in AML. The APL fusion oncoproteins PML-RARα and PLZF-RARα have further been shown to induce increased levels of cyclin A1 mRNA in leukemic cell lines (Muller *et al*., 2000), which suggests that cyclin A1 may be a target for the leukemic fusion proteins.

In this study, we demonstrate that cyclin A1 is localized in the nucleus of normal CD34+ cells, whereas in leukemic blasts from patients and in HL-60 and U-937 leukemic cell lines, cyclin A1 is predominantly cytoplasmic. Relocalization of cyclin A1 protein to the nucleus is induced by treatment with ATRA. In parallel to its nuclear relocalization, cyclin A1 was shown to bind to RARα and CDK1 in an ATRA-dependent manner, which suggested that subcellular localization of cyclin A1 in these cell lines may be mediated by an ATRA-dependent pathway. The subcellular localization of cyclin A1 might be important in the regulation of differentiation of hematopoietic cells, and potentially in the pathogenesis of AML.

Results

Localization of cyclin A1 protein in bone marrow samples from healthy donors and patients using immunohistochemical analysis

Although expression of cyclin A1 has been reported in human hematopoietic progenitor cells (Yang *et al*., 1999), its subcellular localization in normal human hematopoietic cells or in leukemic blasts has not been investigated. We therefore examined the expression of cyclin A1, along with cyclin A2, CDK1, and CDK2, in a large number of normal and leukemic human bone marrow samples by immunohistochemical analysis. To identify the cell-type-specific expression for these proteins, tissue microarrays were simultaneously immunostained for cyclin A1 and either CD34, a marker for hematopoietic progenitors, or CD15 or CD33, cell surface markers specifically for myeloid lineages. The expression of cyclin A1 was restricted to the myeloid progenitors (Figure 1A, panel a). Further, similar to the localization seen in male germ cells (Liu *et al*., 1998), the subcellular distribution of cyclin A1 was predominantly nuclear (Figure 1A, panel a, insert box; Table 1). However, in leukemic blasts from AML patients, the intense staining of cyclin A1 was restricted to the cytoplasmic compartment (Figure 1A, panel b; Table 1). Similar results were obtained with four different anti-cyclin A1 antibodies (data not shown), suggesting the reliability of our observations.

We next compared these results with the staining patterns of cyclin A2, CDK1, and CDK2. Cyclin A2 was both nuclear and cytoplasmic in the normal hematopoietic cells (Figure 1A, panel c) and was also found, but at higher levels, with similar subcellular distribution in leukemic blasts from AML patients (Figure 1A, panel d). The major kinase partners associated with the A-type cyclins, CDK1 and CDK2, were also localized to both the cytoplasm and nucleus in cells from normal bone marrow (Figure 1A, panels e and g) and in leukemic blasts (Figure 1A, panels f

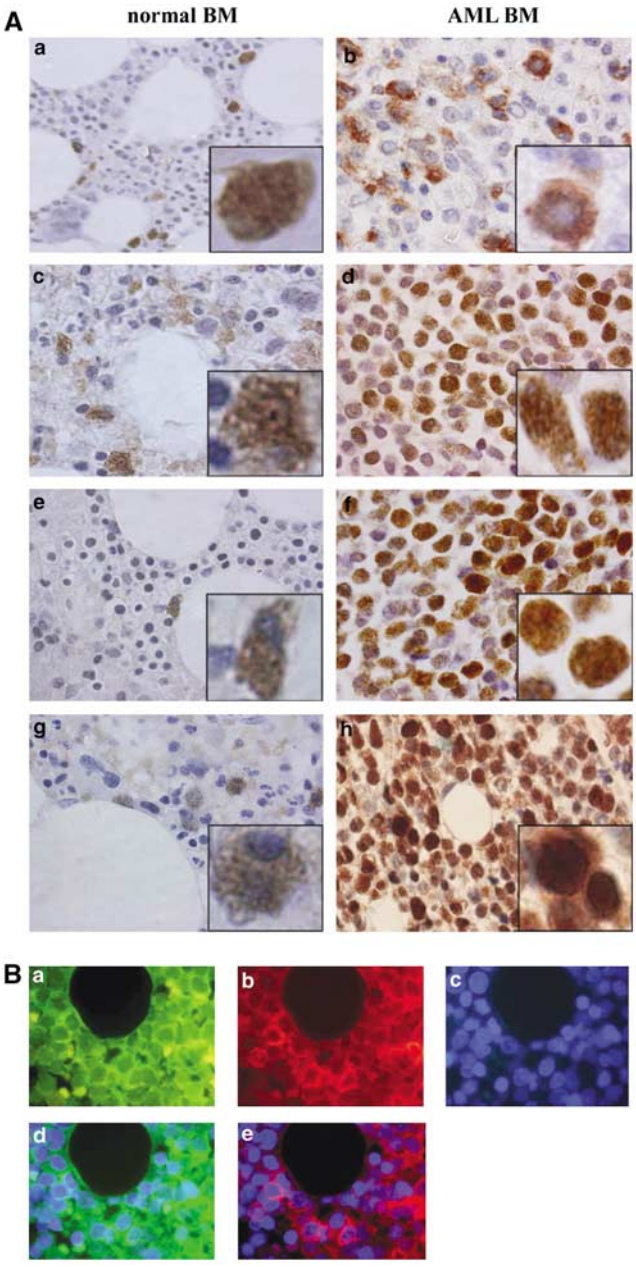

Figure 1 Expression and subcellular localization of cell cycle regulatory proteins in normal and AML bone marrow. (**A**) Immunohistochemical staining of normal (left panels) and AML bone marrow (right panels) using monoclonal anti-cyclin A1 (panels a and b), cyclin A2 (panels c and d), CDK1 (panels e and f) and CDK2 (panels g and h). The photomicrographs were taken under × 100 magnification. Cells in the insert boxes were enlarged from the original pictures. (**B**) Immunofluorescence staining of AML bone marrow using monoclonal anti-cyclin A1 (panel a), anti-CD34 (panel b), and DAPI (panel c). The merged images of panels a and c or b and c are shown in panels d and e. FITC in green, rhodamine in red, and DAPI in blue. The photomicrographs were taken under × 100 magnification

Table 1 Subcellular localization of cyclin A1 protein in bone marrow cells

	Cyclin A1 localization	
Source of bone marrows	*Cytoplasmic*	*Nuclear*
Healthy donors ($n=10$)	Weak in 50% and absent in 50% of cells	Predominantly present in 100% of cells
AML patients ($n=42$)	Predominantly present in 100% of cells	Absent in 90% and weak in 10% of cells
AML patients in remission ($n=25$)	Weak in 50% and absent in 50% of cells	Predominantly present in 100% of cells

Subcellular localization of cyclin A1 in cyclin A1 expressing bone marrow cells from healthy donors, patients with AML, and patients who have achieved complete remission. $n=$ total number of samples in each category

and h). Immunofluorescence analysis was also performed on tissue microarrays and samples were simultaneously stained with antibodies against cyclin A1 and CD34. The double staining further verified that cyclin A1 was predominantly in the nuclei of CD34+ cells from normal bone marrow (data not shown). In contrast, in bone marrow samples from AML patients, cyclin A1 was excluded from the nuclei of leukemic cells (Figure 1B). The subcellular distribution of cyclin A1 was further examined in bone marrow samples from 14 patients in remission. Similar to what was observed in cells from healthy donors, cyclin A1 was found in the nuclei of myeloid cells from patients in remission (Table 1).

Distinct subcellular localization of cyclin A1 in normal hematopoietic progenitor cells and in leukemic cells

To confirm the subcellular distribution of cyclin A1 by an independent method, lysates from normal hematopoietic CD34+ cells, leukemic blasts from an AML patient and cells from leukemic cell lines (U-937 and HL-60) were fractionated into nuclear and cytoplasmic compartments. The presence of cyclin A1, cyclin A2, and CDK2 in each fraction was determined by immunoblot analysis (Figure 2). A cyclin A1 band was observed only in the nuclear fraction of CD34+ cells, while in the leukemic blasts, in which over 95% of cells stained positive for CD34+ antigen (data not shown), cyclin A1 was detected in the cytoplasmic fraction (Figure 2A). This observation further confirmed the results shown by immunohistochemical analysis (Figure 1A and B), where nuclear cyclin A1 was restricted to a subset of hematopoietic progenitors in normal bone marrow and cytoplasmic cyclin A1 was predominant in leukemic blasts in AML patients. This was also the case for leukemic cell lines U-937 and HL-60, although a faint cyclin A1 band was observed in nuclear fraction of U-937 cells (Figure 2B). To further confirm the cytoplasmic localization of cyclin A1 in the cell lines, samples were fixed, embedded in paraffin, sectioned, and immunofluorescence staining was performed with antibodies against cyclin A1 (Figure 2C, panel a) and CD34 (Figure 2C, panel b) and stained with 4′,6-diamidino-2-phenylindole (DAPI) (Figure 2C, panel c). The merged stainings of U-937 cells in Figure 2C, panel d, clearly showed overlap of anti-cyclin A1 and anti-CD34 antibodies. No overlap signal from cyclin A1 and DAPI could be detected, revealing the cytoplasmic localization of cyclin A1.

Cyclin A1 localized to the nuclear compartment in leukemic cells upon ATRA treatment

Retinoic acid induces the terminal differentiation of leukemic cells and further, restores the normal localization of PML and several nuclear body (NB)-associated proteins in NB-4 and U-937 cells (Koken *et al*., 1994; Weis *et al*., 1994). In the present study, we used U-937 and HL-60 cells to determine whether the distinct subcellular localization of cyclin A1 was altered by the treatment with ATRA. Interestingly, ATRA-treatment of U-937 and HL-60 cells resulted in the localization of cyclin A1 to the nuclear compartment of the cells. As shown in Figure 2D, an intense cyclin A1 band was detected in the nuclear fraction of U-937 cells treated with ATRA, in contrast to a barely detectable band in the nuclear fraction from the untreated cells (Figure 2D). Although an increase of total cyclin A1 in response to ATRA treatment was detected, densitometric quantification of the cyclin A1 bands on the immunoblots from three independent experiments revealed that the increase in the nuclear localization of cyclin A1 was significantly more pronounced compared to that of the cytoplasmic localization (data not shown). This suggests that the increased level of cyclin A1 in the nucleus is not due simply to an overall increased level of cyclin A1. Cyclin A2 was nuclear in both control and ATRA-treated cells, although the levels of cyclin A2 declined in the treated cells (Figure 2D). A nuclear localization of cyclin A1 and the presence of cyclin A1/CDK1 complexes in mouse germ cells has been demonstrated previously (Sweeney *et al*., 1996; Liu *et al*., 1998). We were therefore interested in determining the subcellular localization of CDK1 in both control and ATRA-treated cells. In control cells, CDK1 was detected with similar intensity in both cytoplasmic and nuclear fractions. In ATRA-treated cells, CDK1 appeared to be predominantly nuclear, although the total level of CDK1 declined in the treated cells (Figure 2D). The decrease in the levels of cyclin A2 and CDK1 agreed with previous observations in which ATRA treatment resulted in cell cycle arrest in G1/S phase, leading to the downregulation of several cell cycle proteins including

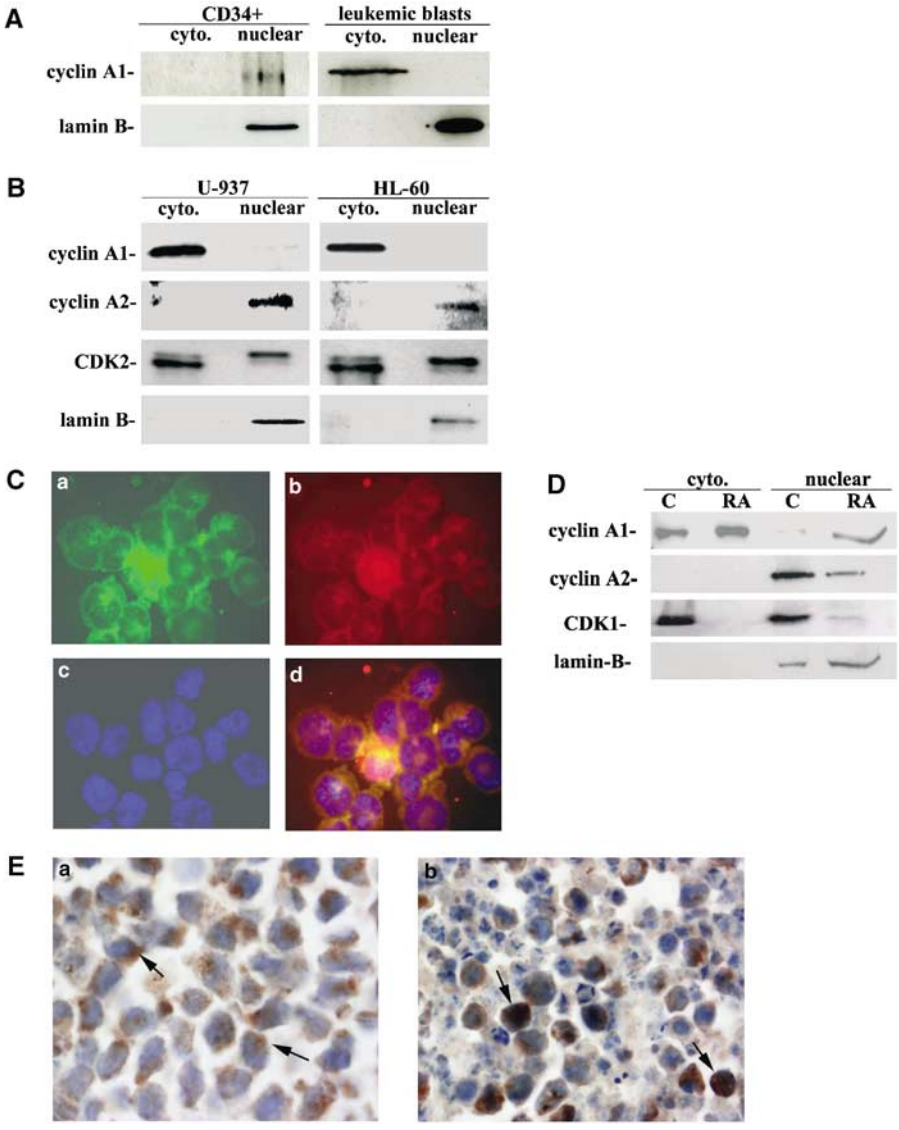

Figure 2 Determination of subcellular localization of cell cycle regulatory proteins in normal hematopoietic cells and in U-937 and HL-60 cell lines. (**A**) CD34+ cells from normal bone marrow and leukemic blasts were isolated. Cytoplasmic (cyto.) and nuclear (nuclear) fractions were analysed by immunoblot analysis using monoclonal antibody against cyclin A1. Lamin B was used as a marker of nuclear protein to verify the quality of the subcellular fractions. (**B**) Fractionated U-937 and HL-60 cell lysates were analysed by immunoblotting using antibodies against cyclin A1 (monoclonal), cyclin A2, CDK2, and lamin B. (**C**) Immunofluorescence analysis of paraffin-embedded U-937 cells: (panel a) FITC-tagged anti-cyclin A1; (panel b) rhodamine-tagged anti-CD34; (panel c) DAPI; and (panel d) merged image of cyclin A1, CD34, and DAPI staining. Photomicrographs were taken at $\times 100$ magnification. (**D**) Immunoblot analysis of subcellularly fractionated cytoplasmic and nuclear compartments from U-937 cells with or without treatment with ATRA. Antibodies to cyclin A1, cyclin A2, CDK1, and lamin B were used as probes as indicated. 'C' indicates control and 'RA' indicates the addition of ATRA. (**E**) Immunostaining detection of U-937 cells with (panel b) or without (panel a) the addition of ATRA using monoclonal cyclin A1 antibody. Arrows indicate cytoplasmic (panel a) and nuclear (panel b) staining of the cells. Pictures were taken at $\times 100$ magnification

cyclin E and cyclin B1 in U-937 cells (Dimberg *et al*., 2002). To further confirm the nuclear versus cytoplasmic localization of cyclin A1, we performed immunostaining of untreated (Figure 2E, panel a) and ATRA-treated U-937 cells (Figure 2E, panel b). Cyclin A1 was indeed detected in the nuclei of many ATRA-treated cells, although cytoplasmic cyclin A1 staining remained (Figure 2E, panel b).

Association of cyclin A1 with CDK1 in ATRA-treated cells

The cytoplasmic localization of cyclin A1 in leukemic cells may affect its association with kinase partners. CDK1 is a major kinase partner for cyclin A1 in mouse germ cells (Liu *et al*., 2000); however, cyclin A1 was reported to form complexes only with CDK2, but not

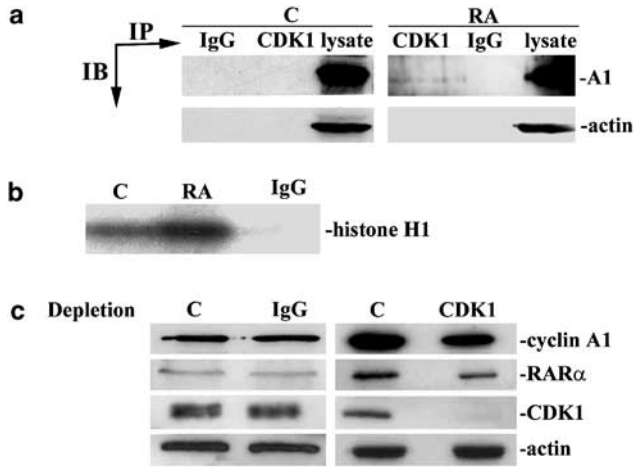

Figure 3 Evaluation of interactions between cyclin A1/CDK1, and the activity of cyclin A1/CDK complexes in U-937 cells. (**a**) Immunoprecipitation (IP) and immunoblot (IB) analysis of untreated cells (C), and ATRA-treated cells (RA). Antibody to CDK1 was used for IP and monoclonal antibody to cyclin A1 was used for IB. Antibody to actin was used as an input loading control. (**b**) Histone H1 kinase activity of cyclin A1/CDK1 complexes. Cyclin A1 immunocomplexes from untreated (C) and ATRA-treated (RA) U937 cells were subjected to kinase assays using histone H1 as substrate. IgG was used as a control in treated and untreated cells. (**c**) Immunodepletion of CDK1 from U-937 cells treated with ATRA. Immunodepleted lysates indicated as 'IgG' or 'CDK1' were subjected to immunoblot analysis together with undepleted control lysates indicated as C. Antibodies to cyclin A1, RARα, and CDK1 were used as probes as indicated. Antibody to actin was used as a loading control

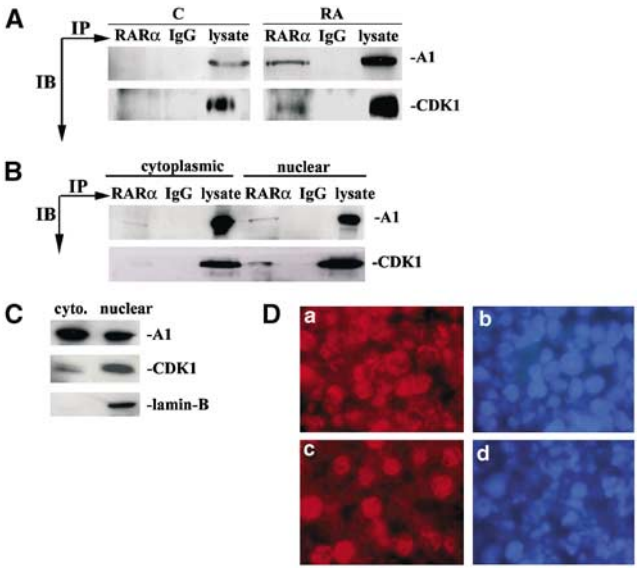

Figure 4 Evaluation of the subcellular distribution of RARα and the interactions between RARα, cyclin A1, and CDK1 in U-937 cells treated with ATRA. (**A**) Antibody to RARα was used for IP of U-937 cells treated with ATRA (RA) or untreated (C) cells. Monoclonal antibodies against cyclin A1 and CDK1 were used for IB. (**B**) Evaluation of localization of cyclin A1/RARα/CDK1 complex. IP and IB were performed on subcellularly fractionated U-937 cells treated with ATRA. RARα was used for IP and antibodies to cyclin A1 and CDK1 were used for IB. IgG was used as control. (**C**) The relative amounts of cyclin A1, CDK1, and lamin B in the lysate used in panel b. (**D**) Immunofluorescence staining of U-937 cells with anti-RARα (left panels) and DAPI (right panels) in untreated (panels a and b) and in ATRA-treated cells (panels c and d)

CDK1, in M-1 leukemic cells (Yang *et al*., 1997). In agreement with these observations, we found cyclin A1/CDK2 complexes (data not shown) but did not detect cyclin A1/CDK1 complexes in untreated U-937 cell lysates (Figure 3a, left panel). In contrast, CDK1 did co-immunoprecipitate with cyclin A1 in U-937 cells treated with ATRA (Figure 3a, right panel). To investigate the activity of cyclin A1-immunoprecipitated complexes in ATRA-treated cells, we next performed kinase assays using histone H1 as substrate (Figure 3b). The phosphorylation of histone H1 was clearly increased in the ATRA-treated sample as compared to the untreated cells. We then examined the effect on cyclin A1 levels when CDK1 complexes were immunodepleted from the lysates of the ATRA-treated cells. A reduction in the level of cyclin A1 was observed (Figure 3c), suggesting that some of the cyclin A1 in the cells was in complex with CDK1 and that the complex formation between cyclin A1 and CDK1 appeared to be ATRA-dependent. This also suggested that cyclin A1/CDK1 complexes may contribute to the increased kinase activity in the ATRA-treated cells. Since RARα is one of the major receptors of ATRA and mediates the response to ATRA in leukemic cells, it was of interest to determine if RARα was present in cyclin A1/CDK1 complexes. As shown in Figure 3c, depletion of CDK1 resulted in a decreased amount of RARα protein as well, indicating that cyclin A1, CDK1, and RARα were in a complex together.

Association of cyclin A1 to RARα may be related to its subcellular localization

The localization of cyclin A1 in the nucleus after treatment with ATRA and the diminution of RARα levels upon immunodepletion of CDK1 led us to determine whether cyclin A1 and RARα may interact directly and whether binding of cyclin A1 to RARα and CDK1 is linked to its nuclear localization. Co-immunoprecipitation experiments were performed using whole cell lysates prepared from U-937 cells, untreated or treated with ATRA (Figure 4A). Both cyclin A1 and CDK1 were detected in the RARα immunocomplex in the cells treated with ATRA (Figure 4A). In contrast, there were no interactions between cyclin A1 and RARα, or CDK1 and RARα in untreated cells (Figure 4A).

We further determined the interaction between cyclin A1 and RARα in the subcellular fractions. ATRA-treated U-937 cells were fractionated into nuclear and cytoplasmic compartments, and the extracted lysates were subjected to co-immunoprecipitation and immunoblot analysis. A complex between cyclin A1 and RARα was found in cytoplasmic and nuclear compartments of the ATRA-treated cells (Figure 4B). Interestingly, CDK1 was also detected in RARα immunocomplex in both nuclear and cytoplasmic compartments (Figure 4B). Cyclin A1 was observed in both subcellular fractions and CDK1 was detected at

higher levels in the nuclear fraction (Figure 4C). This suggested that localization of cyclin A1 to the nucleus was coupled with the formation of a cyclin A1/RARα complex and probably a cyclin A1/CDK1 complex as well. Immunofluorescence analysis verified the presence of RARα in the nucleus and cytoplasm in untreated cells (Figure 4D, panels a and b). In cells treated with ATRA, a more intense staining of RARα was detected in the nuclei (Figure 4D, panels c and d).

Discussion

Previous studies have indicated a strong correlation between elevated levels of cyclin A1 expression and human AML (Yang *et al*., 1997, 1999; Kramer *et al*., 1998). Further, we have reported that transgenic mice overexpressing cyclin A1 in early myeloid cells developed AML (Liao *et al*., 2001). However, the cellular and genetic events underlying the leukemogenesis resulting from the aberrant expression of cyclin A1 remain unknown. An understanding of the oncogenic effect of cyclin A1 requires a precise knowledge of its sites of action. Here, we identified the subcellular distribution of cyclin A1 in normal hematopoietic cells and leukemic cells and provided evidence suggesting that the subcellular distribution of cyclin A1 may be linked to leukemogenesis. The nuclear localization of cyclin A1 in normal CD34+ hematopoietic cells was altered to be predominantly cytoplasmic in leukemic cells from AML patients and in leukemic cell lines. We have demonstrated that cyclin A1 localized to the nuclei of leukemic cell lines, following the treatment of ATRA. We further show that cyclin A1/RARα/CDK1 complexes formed in an apparent ATRA-dependent manner.

The subcellular distribution of cell cycle regulatory proteins including cyclin A2, cyclin B1, and CDK1 is being increasingly recognized as essential for their function (Maridor *et al*., 1993; Yang and Kornbluth, 1999; Moore *et al*., 2003). Previous studies have shown that mammalian cyclin A1, which is required for spermatogenesis, is primarily localized in the nuclei of spermatocytes in mouse (Sweeney *et al*., 1996; Liu *et al*., 1998) and human (Liao *et al*., 2004). Moreover, it plays an essential role for the activation of MPF (cyclin B1/CDK1) during the meiotic cell cycle (Liu *et al*., 1998, 2000), which correlates with a movement of cyclin B1 to the nucleus (Liu *et al*., 2000). In the present study, cyclin A1 was found to exhibit a cell-type-specific expression in normal hematopoietic cells. Further, similar to what was shown in germ cells, the site of subcellular localization of cyclin A1 is the nucleus.

Altered subcellular localization of many key regulatory proteins, including cell cycle regulators, has been linked to malignancy in various types of tumors (Tao and Levine, 1999; Stein *et al*., 2000; Barseguian *et al*., 2002). It has also been reported that altered distribution of p27 is correlated to Barrett's associated adenocarcinoma (Singh *et al*., 1998). A mutant version of cyclin A2, induced by an insertion of human hepatitis B virus, was observed in hepatocellular carcinoma and shown to be predominantly cytoplasmic rather than nuclear (Wang *et al*., 1990; Berasain *et al*., 1998). A direct association between cytoplasmic cyclin A2 and oncogenic transformation was demonstrated in subsequent studies in which a non-viral signal was used to target cyclin A2 to the endoplasmic reticulum, which resulted in several features of cellular transformation (Faivre *et al*., 2002).

In the present study, the alteration of subcellular localization of cyclin A1 correlated with the leukemic phenotype and further, with the absence of complexes between cyclin A1/CDK1. It is possible that cyclin A1 is sequestered away from its normal nuclear site and is thus prevented from exerting its normal function. The subcellular localization of CDKs and their interactions with cyclins are critical factors for determining their specific activity (Moore *et al*., 2003). The ectopic cytoplasmic localization of cyclin A1 might also be correlated with its interactions with the CDK partners. It is not clear whether a cyclin A1/CDK complex may phosphorylate some inappropriate targets in the cytoplasm. However, it has been shown that cellular transformation resulted from the ectopic expression of cytoplasmic cyclin A2 was linked to the aberrant phosphorylation of cyclin A2/CDKs on some substrates (Berasain *et al*., 1998). Our results and previous reported studies suggest a new mechanism of cyclin-related cellular transformation, in which abnormal cytoplasmic localization instead of, or in addition to elevated levels of the proteins are involved in the transformation process.

Interestingly, treatment of ATRA appeared to induce a relocalization of cyclin A1 to the nucleus in U-937 cells. It has been shown that leukemic fusion protein PML-RARα not only displayed the altered cytoplasmic localization itself but also targeted several nuclear proteins such as PML and Sp100 to aberrant sites. Induction of ATRA restored the normal localization of PML (Koken *et al*., 1994; Weis *et al*., 1994). Although it is not clear in the present study what proteins are responsible for the cytoplasmic localization of cyclin A1, a new complex formation between cyclin A1 and RARα upon treatment of ATRA suggests a possible mechanism in which RARα might confer the response to ATRA by promoting cyclin A1 from the cytoplasm to the nucleus. Our finding may be analogous to the altered subcellular localization of PML in APL in response to ATRA treatment (Koken *et al*., 1994; Weis *et al*., 1994).

We observed that the formation of complexes between cyclin A1, CDK1, and RARα appeared to be ATRA-dependent, which suggests that ATRA-mediated pathways are involved. We also showed that redistribution of a significant proportion of cyclin A1 to the nucleus upon ATRA treatment correlated with the formation of complexes between cyclin A1, RARα, and CDK1. We have also demonstrated that cyclin A1/RARα complex is present in both nuclear and cytoplasmic fractions. It has been suggested that targeting of regulatory proteins to specific intracellular sites is mediated by protein–protein interactions, and is critical for their proper

function (Stein *et al*., 2000). Our findings indicate that RARα might be the candidate interacting protein that targets and mediates the localization of cyclin A1 into the nucleus. We further observed cyclin A1/CDK1 complex in ATRA-treated cells in which a significant portion of CDK1 was found in the nuclear fraction. Our results provide a molecular link between subcellular localization of cyclin A1 and ATRA action, and may also suggest that in normal cells, formation of complexes between cyclin A1 and CDK1 or cyclin A1 and RARα is critical.

Materials and methods

Bone marrow samples and sections

For immunohistochemical analysis, bone marrow samples were obtained as formalin-fixed archival specimens from the Department of Pathology, Lund University Hospital in Malmö. They included specimens from 42 patients obtained at the time of diagnosis and which contained 90% leukemic blasts, 14 patients in remission, and 10 normal adults that had been prepared as the tissue microarrays as described previously (Kononen *et al*., 1998). For immunoblotting, bone marrow samples were aspirated from two healthy donors and leukemic cells were isolated from the peripheral blood of an AML patient at the time of diagnosis. Mononuclear cells were purified by gradient centrifugation in Ficoll-paque™ Plus (Amersham Pharmacia Biotech, Uppsala, Sweden). CD34+ cells were isolated using the miniMACS magnetic cell separation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the isolated CD34+ cells was determined by fluorescence-activated cell sorter analysis. For embedding HL-60 and U-937 leukemic cells, cells were fixed in 4% paraformaldehyde, stained with Mayer's hematoxylin, then treated with 70% ethanol, and incubated overnight before being embedded in paraffin and subsequently sectioned.

Source of antibodies

Cyclin A1 antibodies used included a monoclonal antibody (PharMingen, San Diego, CA, USA), goat and rabbit polyclonal antibodies to human A1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and a rabbit polyclonal Ab to mouse A1 (Liu *et al*., 1998). Other antibodies included: monoclonal (PharMingen) and polyclonal (Transduction Laboratories, San Jose, CA, USA) antibodies to human cyclin A2; monoclonal and polyclonal antibodies to human CDK1 and CDK2 (Transduction Laboratories); polyclonal anti-human cyclin B1 and anti-lamin B (Upstate Biotech, New York, NY, USA); and anti-RARα (Santa Cruz) and anti-CD34 (DAKO Glostrup, Denmark).

Immunohistochemistry and immunofluorescence

Paraffin-embedded tissue samples or tissue microarrays were deparaffinized and boiled in 0.01 M citrate buffer, pH 6.0, for 10 min. The staining procedure was performed using a semiautomatic staining machine (Ventana ES, Ventana Inc., Tucson, AZ, USA). The double staining procedure was performed as described previously (Palmqvist *et al*., 2000). In immunofluorescence experiments, incubation with primary antibodies was followed by 1 h incubation with fluorochrome-conjugated antibodies, rhodamine-tagged goat anti-mouse IgG (Chemical International, Temecula, CA, USA) and FITC-conjugated anti-mouse or goat Ig-G (Dako), and Cy3-conjugated anti-mouse Ig-G (Jackson ImmunoResearch, Baltimore, NJ, USA). Finally, the slides were incubated with 1 μM DAPI (Vector Laboratories, Burlingame, CA, USA) for 30 min. The specimens were viewed with a Nikon 800 microscope.

Cell culture and ATRA

Human leukemic cell lines HL-60 and U-937 were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. For ATRA treatment, cells were cultured in the medium containing ATRA (Sigma) dissolved in DMSO/ethanol at a concentration of 100 μM for 48 h. Cells were also treated with 1 μM ATRA for 5 days. The effect of the treatment with 1 μM ATRA on the cells was similar to the short-term treatment with 100 μM ATRA.

Immunoblotting, immunoprecipitation, and kinase assay

For immunoblotting, cells were lysed in ice-cold RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% NP-40, 10 mM NaF, 10 mM phenylmethylsulfonyl fluoride and the protease inhibitor cocktail Complete Mini (Roche, Basel, Switzerland), and centrifuged twice at 12 000 *g* for 30 min at 4°C. For co-immunoprecipitation, 400–2000 μg protein were mixed with 2–3 μg of the appropriate antibodies and 30 μl protein A–sepharose beads or protein G–sepharose beads and then rocked for 2 h at 4°C. The proteins were then separated on SDS–PAGE gels followed by transfer to nitrocellulose membrane. The membranes were probed with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies (Amersham Life Science, Alesbury, UK) and visualized using the Enhanced Chemi-Luminescence detection system (ECL) and ECL films (Amersham Pharmacia Biotech). For antigen immunodepletion, cell lysates were incubated with either CDK1 or IgG antibodies. In kinase assays, cyclin A1-associated complexes were co-immunoprecipitated with monoclonal antibody against cyclin A1 and then washed in RIPA buffer and in EB kinase buffer (50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 20 mM Tris (pH 7.50, 1 mM DTT). The kinase reactions were performed by adding 40 μl EB kinase buffer supplemented with 10 μM ATP, 50 μg/ml calf thymus histone H1 (Upstate Biotech) and 2.5 μCi of [γ-^{32}P]ATP to the beads. After incubation at 30°C for 20 min, the eluted proteins were separated in SDS–PAGE gels and then exposed to autoradiography.

Subcellular fractionation

Cell pellets were resuspended in ice-cold nuclei isolation buffer (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1% Triton X-100), incubated on ice for 10 min, and repelleted. The supernatant containing the cytoplasmic fraction was collected, while the pellet was resuspended in ice-cold RIPA buffer, followed by incubation on ice for 20 min. The lysate was then centrifuged and the supernatant containing the final nuclear fraction was collected. The nuclear and cytoplasmic fractions were subjected to immunoblot analysis as described above.

Acknowledgements
We thank Elise Nilsson and Karl Bacos for the technical assistant, and Stefan Karlsson (Division of Molecular Medicine and Gene therapy, Lund) for helpful discussions. This work was supported by grants from Swedish Cancer Society, Swedish Children's Cancer Foundation, MAS Cancer

Fondation, Kungliga Fysiografiska Sälskapet in Lund, Crafoordska stiftelsen, and MAS foundation (JLP); the Government Public Health Grant and Swegene/WCN (GL), and the NIH (CA09363 and CA95362) (DJW).

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