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The phosphoinositide 3-kinase/Akt pathway regulates cell cycle progression of HL60 human leukemia cells through cytoplasmic relocalization of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ and control of cyclin D_1 expression

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The serine/threonine protein kinase Akt, a downstream effector of phosphoinositide 3-kinase (PI3K), plays a pivotal role in tumorigenesis because it affects the growth and survival of cancer cells. Several laboratories have demonstrated that Akt inhibits transcriptional activation of a number of related forkhead transcription factors now referred to as FoxO1, FoxO3, and FoxO4. Akt-regulated forkhead transcription factors are involved in the control of the expression of both the cyclin-dependent kinase (cdk) inhibitor $p27^{Kip1}$ and proapoptotic Bim protein. Very little information is available concerning the importance of the PI3K/Akt pathway in HL60 human leukemia cells. Here, we present our findings showing that the PI3K/Akt axis regulates cell cycle progression of HL60 cells through multiple mechanisms also involving the control of FoxO1 and FoxO3. To this end, we took advantage of a HL60 cell clone (HL60AR cells) with a constitutively activated PI3K/Akt axis. When compared with parental (PT) HL60 cells, HL60AR cells displayed higher levels of phosphorylated FoxO1 and FoxO3. In AR cells forkhead factors localized predominantly in the cytoplasm, whereas in PT cells they were mostly nuclear. AR cells proliferated faster than PT cells and showed a lower amount of the cdk inhibitor $p27^{Kip1}$, which was mainly found in the cytoplasm and was hyperphosphorylated on threonine residues. AR cells also displayed higher levels of cyclin D1 and phosphorylated p110 Retinoblastoma protein. The protein levels of cdk2, cdk4, and cdk6 were not altered in HL60AR cells, whereas the activities of both ckd2 and cdk6 were higher in AR than in PT cells. These results show that in HL60 cells the PI3K/Akt signaling pathway may be involved in the control of the cell cycle progression most likely through mechanisms involving the activation of forkhead transcription factors.
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Introduction

The serine/threonine protein kinase Akt/protein kinase B (PKB) is a pleckstrin-homology domain containing serine/threonine kinase.[1,2] Akt acts downstream of phosphoinositide 3-kinase (PI3K). Therefore, Akt is activated upon treatment of cells with a variety of extracellular growth factors that stimulate PI3K and production of 3′ phosphoinositide lipids.[3,4] Several lines of evidence suggest that PI3K regulates Akt activation through the binding of phosphatidylinositol 3,4,5 trisphosphate ($PtdIns(3,4,5)P_3$) to the pleckstrin homology domain of Akt, resulting in the recruitment of Akt to the cell membrane. A conformational change of Akt then follows, allowing residues Thr 308 and Ser 473 to be phosphorylated by upstream kinases (reviewed in Brazil and Hemmings[5]). Several downstream targets of Akt have been identified, pointing to the possible mechanisms by which Akt promotes cell proliferation and survival and blocks apoptosis. Akt phosphorylates and inactivates proapoptotic proteins such as Bad and procaspase-9.[2] Akt activates NF-κB transcription factor that controls the expression of several antiapoptotic proteins such as FLIP, XIAP, survivin, and IAP-2 [eg Mitsiades *et al*[6]]. Another target of Akt is CREB transcription factor which stimulates transcription of survival genes such as Bcl-2 and Mcl-1.[2,7,8] Furthermore, several laboratories have demonstrated that Akt inhibits transcriptional activation of a number of related transcription factors (FKHR/FKHRL1/AFX),[9–11] now referred to as FoxO1, FoxO3, and FoxO4.[12] Each of these forkhead factors contains phosphorylation sites for Akt, and Akt-mediated phosphorylation was shown to result in translocation of these factors from the nucleus to the cytoplasm.[10,13] Akt-regulated forkhead transcription factors are involved in the control of the expression of both the cyclin-dependent kinase (cdk) inhibitor $p27^{Kip1}$ and Bim, a proapoptotic member of the Bcl-2 family.[14] These findings indicate that forkhead factors play a key role in the regulation of cell cycle progression and apoptosis. Owing to its multiple targets, Akt is thought to play a fundamental role in tumorigenesis.[1]

The PI3K/Akt pathway has been investigated in a large variety of cell lines. Surprisingly, however, very little information is available concerning the importance of this pathway in HL60 cells. These cells originated from a human promyelocytic leukemia and are a useful model for studying signal transduction networks that which are critical for survival and proliferation of leukemic cells. We have recently described a HL60 human leukemia cell clone (named HL60AR, for apoptosis resistant) with a constitutively activated PI3K/Akt axis.[15] Subsequently, we have shown that HL60AR cells are resistant to multiple chemotherapeutic drugs and all-*trans*-retinoic acid (ATRA).[16] Drug resistance could be lowered by inhibitors of the PI3K/Akt signaling pathway. The activation of the PI3K/Akt pathway in AR cells is due to an autocrine production of insulin-like growth factor-1, a powerful stimulator of this signaling network.[16] We further demonstrated that this clone is resistant to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL),[17] one of the latest members of the TNF superfamily known to induce apoptosis in a wide variety of cancer cells.[18,19] TRAIL resistance of HL60AR cells was dependent on NF-κB activation and pharmacological inhibitors of PI3K/Akt pathway restored TRAIL sensitivity of this clone.[17] These results pointed to a key role played by the PI3K/Akt pathway in determining resistance to conventional (chemotherapy, ATRA) or novel (TRAIL) therapeutic treatments.

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Since we observed that HL60AR cells grew faster than parental (PT) HL60 cells, we felt they could constitute a useful model to investigate the relevance (if any) of the PI3K/Akt signaling in the control of the cell cycle progression of HL60 cells. Indeed, evidence collected from other cell types points to a key role played by the PI3K/Akt pathway in governing the molecular mechanisms necessary for cell cycle progression.[4]

Here, we show that HL60AR cells displayed a hyperphosphorylation of FoxO1 and FoxO3 transcription factors as well as their translocation from nucleus to cytoplasm. AR cells also showed a reduced G1 phase length. This was accompanied by a lowered synthesis of the cdk inhibitor $p27^{Kip1}$, which also relocalized to cytoplasm and was hyperphosphorylated on threonine residues. In addition, we detected an increase in the amount of cyclin D1 and an enhanced phosphorylation of the p110 Retinoblastoma (Rb) protein in HL60AR cells.

Materials and methods

Chemicals and reagents

Bovine serum albumin (BSA), propidium iodide (PI), RNase A, RPMI 1640, fetal calf serum, normal rabbit IgG, normal mouse IgG, normal goat serum, fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG, and peroxidase-conjugated secondary antibodies were from Sigma (St Louis, MO, USA). The COMPLETE Protease Inhibitor Cocktail, and the Lumi-Light^{Plus} enhanced chemiluminescence (ECL) detection kit were from Roche Applied Science (Milan, Italy). Calf thymus histone H1, wortmannin and Ly 294002 were from Calbiochem (La Jolla, CA, USA). The Akt kinase nonradioactive assay kit and glutathione-*S*-transferase (GST)-Rb-(769–921) fusion protein were from Cell Signaling Technology (Beverly, MA, USA). Protein A/G-Agarose was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). [γ-^{32}P]ATP was from Amersham Pharmacia Biotech (Uppsala, Sweden). Colloidal gold-conjugated secondary antibody was from British Biocell International (Cardiff, UK). The following primary antibodies were used in this study. From Upstate Biotechnology (Lake Placid, NY, USA): rabbit polyclonals to total FoxO3, and to Thr 32 phosphorylated FoxO3 (p-FoxO3). From Santa Cruz Biotechnology: rabbit polyclonals to cyclin D1, D2, D3, E, cdk2, cdk4, and cdk6. From Calbiochem: mouse monoclonal to human lamin B. From Cell Signaling Technology (Beverly, MA, USA): rabbit polyclonals to Thr 473 phosphorylated Akt (p-Akt, Catalog #9271, specific for Western blot and immunoprecipitation), to Ser 473 p-Akt (Catalog #9277, specific for immunohistochemistry), total Rb protein, to Ser 780 phosphorylated Rb protein (p-Rb protein), to Ser 795 p-Rb protein, to Ser 807/811 p-Rb protein, to total FoxO1, to Ser 256 phosphorylated FoxO1 (p-FoxO1). From Sigma: mouse monoclonal to β tubulin. From BD Transduction Laboratories (San Diego, CA, USA): mouse monoclonal to $p27^{Kip1}$ (clone 57). From Alexis Biochemicals (Lausen, Switzerland): monoclonal antibody to phosphothreonine (clone 14B3).

Cell culture

HL60PT (for parental, obtained from the American Type Culture Collection-CCL 240) and HL60AR (for Apoptosis Resistant) cells were routinely maintained in RPMI 1640 supplemented with 10% fetal calf serum at an optimal cell density of $3–8 \times 10^5$ cells/ml. The medium was changed every 3 days. All the results presented in this article have been obtained with cells cultured for 2 days, unless otherwise indicated. In some experiments, cells had been preincubated for 24 h with nontoxic concentrations of the PI3K pharmacological inhibitors wortmannin (0.1 μM) or Ly294002 (10 μM).

Cell cycle analysis and determination of the G1 phase length

To evaluate the cell cycle, 5×10^5 cells were fixed in 1 ml cold 70% ethanol at 4°C for 1 h. The cells were centrifuged, washed in PBS, resuspended in 0.4 ml PBS, and treated with 0.5 μg/ml RNAse A for 1 h at 37°C. After addition of 20 μg/ml of PI, samples were analyzed with an Epics XL flow cytometer equipped with the appropriate software (Beckman-Coulter Immunology, Miami, FL, USA). The G1 length was estimated by determining the doubling time and the proportion of cells in the G1 phase. The cell number was determined at 0, 24, 48, and 72 h in culture and plotted semilogarithmically *vs* the time, and the doubling time was calculated from the slope of the straight line thus obtained. The proportion of cells in the G1 phase was derived from the average percentage of cells in the G1 phase, measured by flow cytometry. The length of the G1 phase was calculated using the formula: $T_{G1}/T_C = 1n\ (F_{G1}+1)/\ln 2$ (T_C is the doubling time, T_{G1} is the duration of G1 and F_{G1} is the fraction of cells in G1). In these experiments, when required, the PI3K inhibitors were added to the culture media every 24 h.

Immunoprecipitation

Cells or nuclei were resuspended in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, supplemented with the COMPLETE Protease Inhibitor Cocktail and phosphatase inhibitors (5 mM sodium pyrophosphate, 2 mM β-glycerophosphate, 1.0 mM Na_3VO_4, and 20 nM okadaic acid), then homogenized by 30 passages through a 25 gauge needle. Cell homogenates (1 ml, containing 500 μg of protein) were precleared by adding 5 μg of normal rabbit or mouse IgG and 10 μg of 50% Protein A/G agarose, followed by incubation for 1 h at 4°C and centrifugation at 12 000 *g* for 10 min at 4°C. Then, the samples were incubated for 2 h at 4°C under constant agitation with 5 μg of the antibody of interest. A 10 μg measure of 50% Protein A/G Agarose was then added and the incubation was proceeded for 1 h at 4°C under constant agitation.

Isolation of nuclei

Briefly, cells were washed once in PBS, pH 7.4 (without Ca^{2+} and Mg^{2+}) and resuspended to 1.5×10^7/ml in 10 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml each of aprotinin and leupeptin, 5 mM sodium pyrophosphate, 2 mM β-glycerophosphate, 1.0 mM Na_3VO_4, and 20 nM okadaic acid. After 5 min at 0°C, cells were sheared by six passages through a 22 gauge needle fitted to a 30 ml plastic syringe. The concentration of Mg^{2+} was brought to 5 mM and nuclei were sedimented at 400 *g* for 6 min. The postnuclear supernatant was saved for analysis of cytoplasmic fraction. Nuclei were washed once in 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and resuspended to 2 mg DNA/ml in 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 5 mM $MgCl_2$. This procedure allowed the preparation of nuclei free of cytoplasmic contamination as demonstrated by transmission electron microscopy analysis (data not shown).

Preparations of extracts for Western blot analysis

Cells or nuclei were resuspended at $\sim 10^7$/ml in boiling lysis buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecylsulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, and the protease and phosphatase inhibitor cocktail. Lysates were briefly sonicated to shear DNA and reduce viscosity, boiled for 5 min to solubilize protein, and stored at −80°C until required.

Western blot analysis

Protein (50–80 μg) separated on SDS-polyacrylamide gels (SDS-PAGE) was transferred to nitrocellulose sheets using a semidry blotting apparatus. Sheets were saturated in PBS containing 5% normal goat serum and 4% BSA for 60 min at 37°C (blocking buffer), then incubated overnight at 4°C in blocking buffer containing the primary antibodies. After four washes in PBS containing 0.1% Tween-20, they were incubated for 30 min at room temperature with the appropriate peroxidase-conjugated secondary antibodies, diluted 1:5000 in PBS-Tween-20, and washed as above. The bands were visualized by the ECL method.

Akt kinase assay

This was performed according to manufacturer's instructions. Briefly, in this assay a polyclonal antibody was used to selectively immunoprecipitate Akt from cell or nuclear lysates. The resulting immunoprecipitates were incubated with GSK-3 fusion protein in the presence of ATP. This allowed Akt to phosphorylate GSK-3. Phosphorylation of GSK-3 was measured by Western blotting using an antibody to Ser 21/9 phosphorylated GSK-3α/β (p-GSK-3α/β).

Immunocomplex kinase assay

Cells were suspended in lysis buffer containing 50 mM Hepes-NaOH, pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 0.1% Tween-20, 10% glycerol, 1 mM PMSF, 10 μg/ml each of aprotinin, leupeptin, and pepstatin A, 5 mM sodium pyrophosphate, 2 mM β-glycerophosphate, 20 mM NaF, 1.0 mM Na_3VO_4, and 20 nM okadaic acid, followed by a 10-s sonication and clearing by centrifugation at 14 000 rpm in a microfuge for 15 min. Supernatants were assayed for protein concentration and protein samples of 4 mg each were incubated for 4 h at 4°C with appropriate polyclonal antibodies (5 μg), then immunoprecipitated for 1 h with 10 μg of 50% Protein A/G agarose. Immunoprecipitated proteins on beads were washed three times with 1 ml of lysis buffer and twice with kinase reaction buffer (50 mM Hepes-NaOH, pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 10 μg/ml each of aprotinin, leupeptin, and pepstatin A, 0.2 mM PMSF, 20 mM NaF, 0.2 mM Na_3VO_4). The beads were incubated for 30 min with 40 μl kinase reaction buffer supplemented with 10 μM ATP, 0.4 mCi/ml [γ-^{32}P]ATP, and 40 μg substrate/ml. Histone H1 was used as kinase substrate for cdk2, and GST-Rb was used as kinase substrate for both cdk4 and cdk6. After incubation at 30°C for 30 min, the reaction was stopped by adding 3 × boiling electrophoresis sample buffer. The supernatant was separated SDS-PAGE and the radioactivity was detected by autoradiography.

Immunoelectron microscopy detection of Ser 473 p-Akt

A postembedding technique was used as a method for transmission immunoelectron microscopy. Samples were fixed with 1% glutaraldehyde in sodium phosphate buffer, pH 7.3, for 35 min at 4°C, dehydrated up to 70% ethanol and embedded in London Resin White. Polymerization was performed overnight at 4°C by accelerator. To block nonspecific binding sites, the grids were treated with TBS buffer (20 mM Tris-HCl, pH 8.2, 150 mM NaCl, 0.1% BSA, 0.05% Triton, 0.05% Tween-20) for 10 min at room temperature. Sections were incubated at 4°C with the primary antibody (anti-Ser 473 p-Akt, Catalog #9277) diluted 1:50 in TBS. Grids were washed several times in TBS and then incubated with the secondary antibody (1:50 in TBS). The secondary antibody was an anti-rabbit IgG conjugated with 15 nm colloidal gold particles. The grids were briefly stained with uranyl acetate (5 min) and subsequently with lead citrate (3 min). Sections were examined with a JEOL-JEM 100S electron microscope.

Immunofluorescent staining

Cells in media were plated onto 0.1%. poly-L-lysine-coated glass slides, and adhesion was allowed to proceed for 30 min at room temperature. Samples were fixed in freshly prepared 4% paraformaldehyde in PBS for 30 min at room temperature and then permeabilized with 0.2% Triton X-100 for 10 min. After several washes with PBS, nonspecific binding of antibodies was blocked by a 30 min incubation at 37°C with PBS, 2% BSA, 5% normal goat serum (NGS). Slides were then incubated for 3 h at 37°C with either polyclonal antibody to Ser 473 p-Akt (specific for immunocytochemistry) or monoclonal antibody to $p27^{Kip1}$ (both diluted 1:50 in PBS, 2% BSA, 5% NGS), then washed three times in PBS and reacted with FITC-conjugated anti-rabbit or anti-mouse IgG, diluted 1:200 in PBS, 2% BSA, 5% NGS for 1 h at 37°C. Samples were subsequently washed three times in PBS, stained with 0.01 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in PBS and mounted in 20 mM Tris-HCl, pH 8.2, 90% glycerol containing 2.3% of the antifading agent 1,4-diazobicyclo–octane. Slides were observed and photographed using a Zeiss Axiophot epifluorescence microscope.

Results

Ser 473 p-Akt mainly localizes to the nucleus of HL60AR cells

We recently described a HL60 cell clone (named HL60AR) with a constitutively active PI3K/Akt pathway.[15] When compared with HL60PT cells, the AR clone displays higher levels of phosphorylated (active) Akt.[16] Since previous investigations have highlighted that p-Akt may migrate to the nucleus (reviewed in Neri *et al* [20]), we sought to analyze the subcellular distribution of Ser 473 p-Akt in HL60 PT and AR cells. As shown in Figure 1a, Western blot analysis showed that a nearly similar amount of Ser 473 p-Akt was detectable in the cytoplasmic fraction prepared from either cell type. On the other hand, no Ser 473 p-Akt immunoreactivity could be detected in nuclei obtained from PT cells, whereas a strong immunoreactivity for Ser 473 p-Akt was detected in the nuclear fraction prepared from HL60AR cells. We next performed an *in vitro* Akt kinase assay using both the cytoplasmic and the nuclear fraction. As presented in Figure 1b, c, a similar amount of cytoplasmic

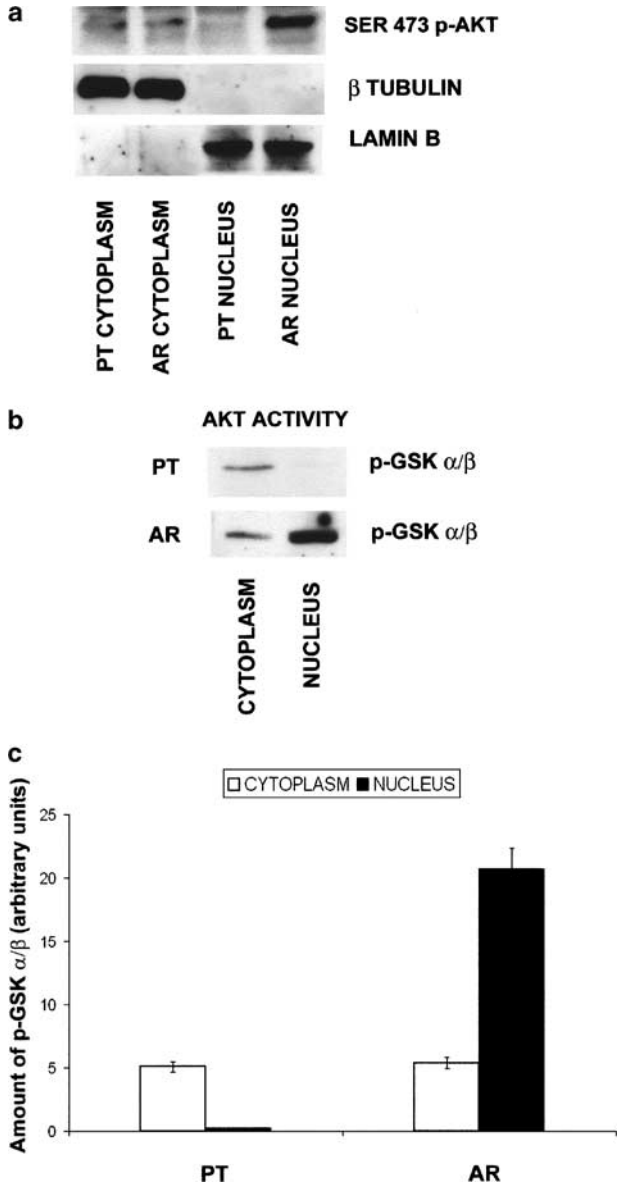

Figure 1 Ser 473 p-Akt preferentially localizes to the nucleus of HL60AR cells. Panel (a) Western blotting analysis for Ser 473 p-Akt (active Akt) performed on cytoplasmic and nuclear fractions prepared from HL60PT and AR cells. To detect Ser 473 p-Akt antibody #9271 was employed. Blots were probed with antibody to β tubulin or to lamin B to ensure equal protein loading and purity of the fractions. Protein (80 μg), separated by SDS-PAGE, was blotted to each lane. Reactive bands were revealed by ECL. A blot representative of three separate experiments is shown. Panel (b) Akt activity assay on GSKα/β performed on cytoplasmic and nuclear fractions prepared from HL60PT and AR cells. Immunoprecipitation of cytoplasm or nuclear fractions was performed with an antibody to total Akt. The immunoprecipitates were subjected to an *in vitro* kinase assay using GSK α/β as a substrate (1 μg). The reaction products were resolved on a 12% SDS-PAGE and subjected to Western blot analysis using anti-p-GSK α/β antibody. One representative experiment of three is shown. Panel (c) relative amount of p-GSKα/β, determined by densitometric scanning of the films. Results are mean $\pm$ s.d. of three different experiments.

activity was detected in either HL60PT or AR cells. However, when the nuclear Akt kinase activity was assayed, it turned out to be much higher in AR than in PT cells. To further demonstrate that Ser 473 p-Akt was selectively concentrated in the nucleus of HL60AR cells, we employed both immunofluorescence and immunoelectron microscopy analysis. As shown in Figure 2a, immunofluorescent staining revealed that Ser 473 p-Akt was detectable only in the nucleus of AR cells (compare picture a with c). Immunogold labeling followed by transmission electron microscope analysis demonstrated that in HL60PT cells, a scarce immunolabeling for Ser 473 p-Akt was

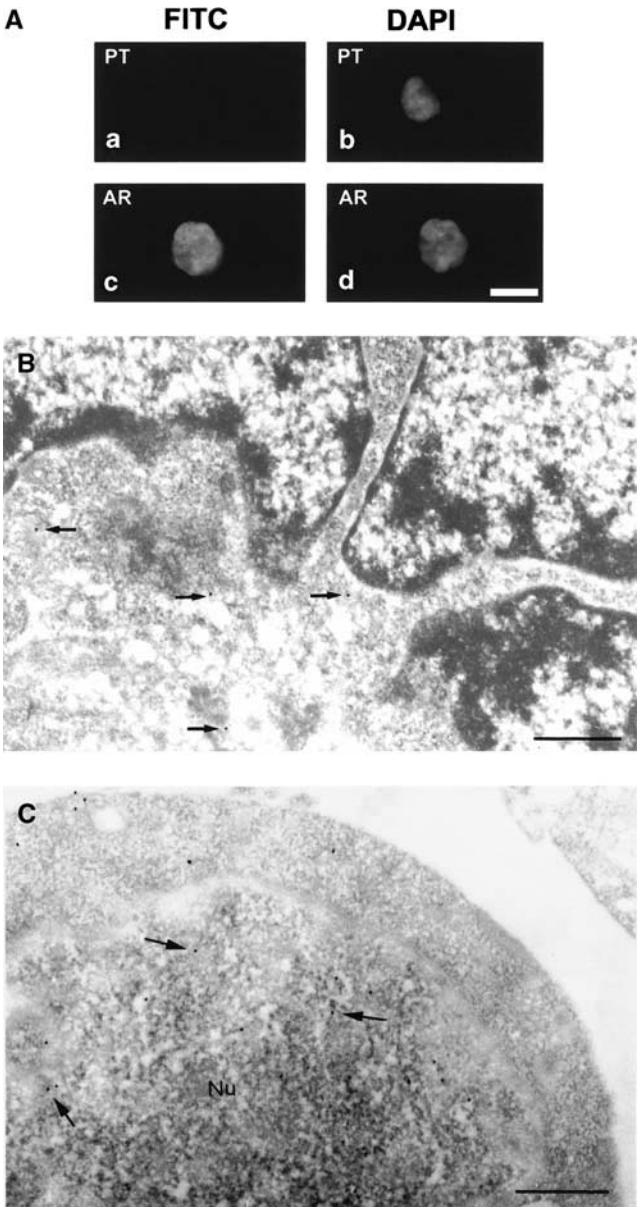

Figure 2 Immunolocalization of Ser 473 p-Akt in HL60PT and AR cells. Panel (A) immunofluorescence staining for Ser 473 p-Akt. Cells adherent to coverslips were fixed, permeabilized and stained with an immunocytochemistry-specific polyclonal antibody to Ser 473 p-Akt, which was then revealed by a FITC-conjugated anti-rabbit IgG. Samples were counterstained for DNA by DAPI. Note that the exposure time of (a) and (c) was chosen to evidentiate nuclear staining, if present. For this reason, the faint cytoplasmic staining is not visible. Scale bar: 5 μM. (B) immunoelectron microscopy analysis for Ser 473 p-Akt in HL60PT cells. (C) immunoelectron microscopy analysis for Ser 473 p-Akt in HL60AR cells. In (B) and (C), the arrowheads point to gold particles. Nu: nucleus. Scale bar in (B and C) 0.5 μm.

present only in the cytoplasm. In contrast, in HL60AR cells, besides some gold particles scattered in the cytoplasm, there was a strong immunoreactivity localized to the nucleus (Figure 2b).

FoxO1 and FoxO3 are hyperphosphorylated in HL60AR cells

Akt is known to directly phosphorylate a number of forkhead transcription factors belonging to the FoxO subfamily. Phosphorylation of these forkhead factors results in their exclusion from the nucleus and a subsequent inhibition in transcriptional activation of forkhead target genes. Therefore, we wanted to analyze whether, also in HL60 cells, regulation of FoxO activity is mediated by PI3K/Akt signaling. In particular, we focused on both FoxO1, which can be phosphorylated by Akt on Ser 256, and FoxO3, which can be phosphorylated on Thr 32 by Akt. As presented in Figure 3a, Western blots showed that the amount of either total FoxO1 or total FoxO3 was similar in HL60PT and AR and was not affected by treatment of AR cells with two unrelated pharmacological inhibitors of the PI3K/Akt axis, wortmannin and Ly294002, used at concentrations that promote inhibition of Akt activity.[16] In contrast, both Ser 256 p-FoxO1 and Thr 32 p-FoxO3 were more abundant in AR than in PT cells and were sensitive to either wortmannin or Ly294002. We then performed Western blot analysis of FoxO1 and FoxO3 in subcellular fractions. The results presented in Figure 3b indicated that in PT cells both the transcription factors were predominantly localized in the nucleus, whereas in AR cells they were exclusively (FoxO1) or mostly (FoxO3) cytoplasmic. In AR cells treated with wortmannin or Ly294002, the localization of the examined FoxO factors was essentially nuclear (Figure 3b).

Cell cycle and G1 length analyses

It is well established that the PI3K/Akt pathway controls cell cycle progression by a number of mechanisms (reviewed in Katso *et al*;[4] Lawlor and Alessi[21]). We, therefore, set out to analyze by flow cytometry the cell cycle of HL60PT and AR cells. As presented in Figure 4, in the HL60AR clone there was, after 48 h of culture, a marked increase in the quantity of cells in either S or G2/M phase of the cell cycle when compared with PT cells. In contrast, the percentage of cells in the G1 phase of the cycle was higher in PT than in AR cells. Treatment of AR cells with wortmannin or Ly294002 resulted in a dramatic decrease of cells in S and G2/M, whereas those in G1 phase increased. Moreover, as shown in Figure 5, the G1 transit time was shorter in AR cells than in PT cells. Exposure of HL60AR cells to PI3 K inhibitors considerably slowed G1 transit.

The PI3K/Akt axis influences p27^{Kip1} expression, localization, and threonine phosphorylation in HL60 cells

Previous results have shown that p27^{Kip1} expression is controlled by FoxO3 in an Akt-dependent fashion.[14] p27^{Kip1} is a well-known regulator of the G1/S transition through its cdk inhibitory activity, which blocks the cells in G1 phase of the cell cycle by preventing cdk-dependent phosphorylation of Rb protein.[22,23] To study whether p27^{Kip1} expression is regulated in a PI3K/Akt-dependent manner also in HL60 cells, we analyzed p27^{Kip1} protein levels in HL60PT, HL60AR, and in

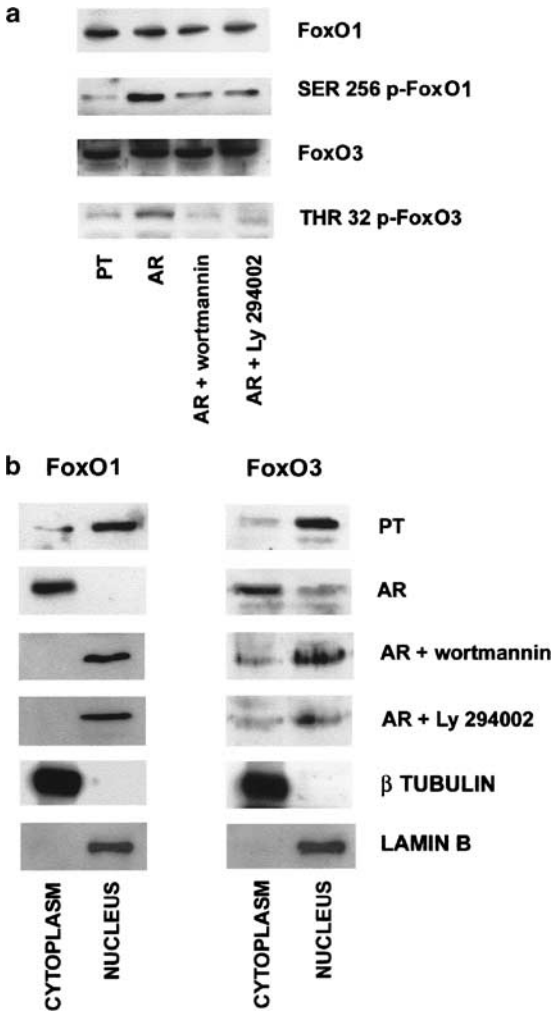

Figure 3 The activity and localization of forkhead transcription factors are regulated via the PI3K/Akt pathway. (a) Western blot analysis for FoxO1, Ser 256 p-FoxO1, FoxO3, and Thr 32 p-FoxO3 in HL60 PT and AR cells, and in AR cells treated with wortmannin or Ly294002. (b) Western blot analysis showing subcellular distribution of FoxO1 and FoxO3 in HL60 PT and AR cells, and in AR cells treated with wortmannin (0.1 μM) or Ly294002 (10 μM) for 24 h before harvesting. In both (a) and (b) 80 μg of protein was blotted to each lane. In (b), the purity of the fractions was evaluated by immunochemical analysis with antibody to β tubulin and lamin B. The blots represent prototypical examples of experiments replicated at least three times.

HL60AR cells treated with PI3K pharmacological inhibitors. In Figure 6a, we demonstrate by Western blot analysis a down-regulation of p27^{Kip1} in AR cells when compared with PT cells. Treatment of AR cells with wortmannin or Ly294002 resulted in higher levels of p27^{Kip1}. Very recent findings, obtained in breast cancer cells, have shown that Akt also phosphorylates p27^{Kip1} on Thr 157 and promotes its cytoplasmic relocalization from the nucleus.[24,25] We, therefore, sought to investigate whether or not this type of regulation might be effective also in HL60 cells. To this end, we first immunoprecipitated p27^{Kip1} from whole-cell lysates, blotted it to nitrocellulose paper and probed the blots with a monoclonal antibody that selectively recognizes phosphothreonine. As shown in Figure 6b, the immunoreactivity for phosphothreonine was much higher in HL60AR cells than in PT cells, even if the levels of immunoprecipitated total p27^{Kip1}

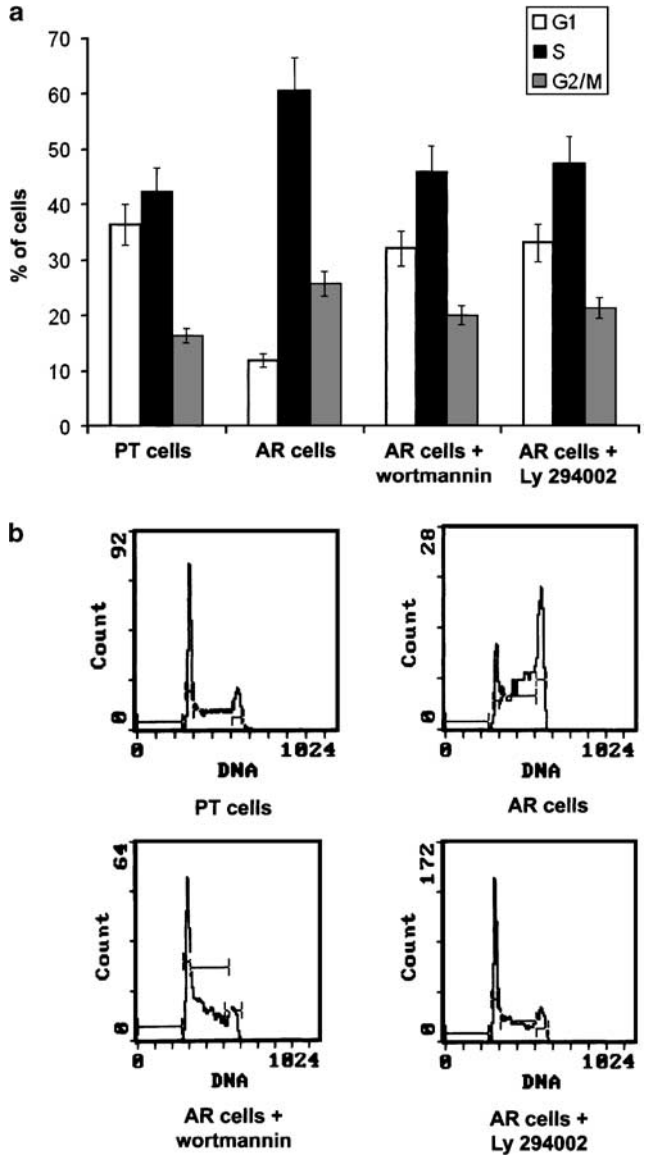

Figure 4 Cell cycle is affected by the PI3K/Akt pathway. (a) Cells cultured for 48 h in medium after seeding were fixed by ethanol, stained with PI and analyzed by flow cytometry. Wortmannin (0.1 μM) or Ly294002 (10 μM) was present for 24 h before fixation. Note that cells in sub-G1 (apoptotic) are not represented. Results are mean $\pm$ s.d. of three different experiments. (b) Representative histograms showing flow-cytometric analysis of PI-stained samples.

were much lower in AR than in PT cells. If HL60AR cells had been pretreated with either wortmannin or Ly294002, the levels of threonine phosphorylation of $p27^{Kip1}$ were very low. In Figure 6c we present data obtained by subcellular fractionation experiments. It was clear that in HL60PT cells, $p27^{Kip1}$ was mainly intranuclear, while in AR cells it was more abundantly expressed in the cytoplasm. Treatment of AR cells with either wortmannin or Ly294002 resulted in $p27^{Kip1}$ being mostly nuclear.

Since the cytoplasmic relocalization of $p27^{Kip1}$ detected in AR cells might simply be the consequence of an increased extractability of the protein from nuclei during cell fractionation, we performed *in situ* immunocytochemical fluorescence analysis of $p27^{Kip1}$ localization. As presented in Figure 7, $p27^{Kip1}$ was essentially nuclear in HL60PT cells, whereas it was mostly cytoplasmic in HL60AR cells. Treatment of HL60AR cells with wortmannin of Ly294002 restored the nuclear localization of the protein.

The PI3KAkt pathway controls cyclin D1 expression and p-Rb levels in HL60 cells

Evidence is now accumulating that the PI3K/Akt axis is required for enhanced expression of cyclin D1, which is essential for cdk-activation and thus for G1 progression.[4,26] PI3K/Akt-evoked cyclin D1 upregulation may occur through multiple mechanisms, including enhanced mRNA synthesis and stability, increased mRNA translation, and stabilization of cyclin D1 protein (reviewed in Katso *et al*[4]). We, therefore, investigated whether cyclin D1 expression was increased in HL-60AR cells. We analyzed cyclin D1 at the level of protein by Western blot. Indeed, the amount of cyclin D1 was higher in AR than in PT cells and was sensitive to PI3K pharmacological inhibitors (Figure 8). In contrast, we did not detect differences between HL60PT and AR cells as far as the expression of cyclin D2, cyclin D3, cyclin E, cdk2, cdk4, and cdk6 was concerned. However, AR cells contained higher amount of Ser 780 p-Rb, Ser 795 p-Rb, and Ser 807/811 p-Rb when compared with PT cells, whereas the quantity of total Rb protein was unchanged in the two types of cells. Phosphorylation of Rb protein in these three sites was highly sensitive to wortmannin or Ly294002.

Cdk activities

We next measured cdk activities in HL60PT and AR cells. To evaluate ckd2 activity, we immunoprecipitated cyclin E. As shown in Figure 9, the amount of immunoprecipitable cyclin E was unchanged in PT and AR and was not sensitive to wortmannin or Ly294002. However, when the immunoprecipitates were probed with an antibody to $p27^{Kip1}$ it became evident that the amount of this protein bound to cyclin E was much lower in AR than in PT cells. Treatment of AR cells with PI3 K inhibitors raised the amount of $p27^{Kip1}$ associated with cyclin E. Cdk2 activity was determined in the immunoprecipitates by phosphorylation of histone H1, one of its principal substrates. As shown in Figure 9, AR cells had higher cdk2 activity than PT cells. Cdk2 activity of AR cells was markedly sensitive to wortmannin or Ly294002. The activity of both cdk4 and cdk6 were assayed on immunoprecipitates using GST-Rb as phosphorylation substrate. As shown in Figure 9, the activity of cdk 4 was similar in PT and AR cells and was not sensitive to inhibitors of the PI3K/Akt pathway. In contrast, cdk6 activity was higher in AR than in PT cells and could be downregulated by treatment with the pharmacological inhibitors.

Discussion

This study demonstrates for the first time that the control of two members of the forkhead family of transcription factors, FoxO1 and FoxO3, is mediated by the PI3K/Akt axis in HL60 human leukemia cells, and it is likely involved in the regulation of the cell cycle progression. To reach such a conclusion, we took advantage of a HL60 cell clone, isolated in our laboratory, with an upregulated PI3K/Akt pathway. In HL60AR cells, Ser 473 p-Akt mainly localized to the nucleus, as demonstrated by Western blot analysis and *in vitro* kinase assay performed on a highly purified nuclear fraction, and *in situ* immunolabeling.

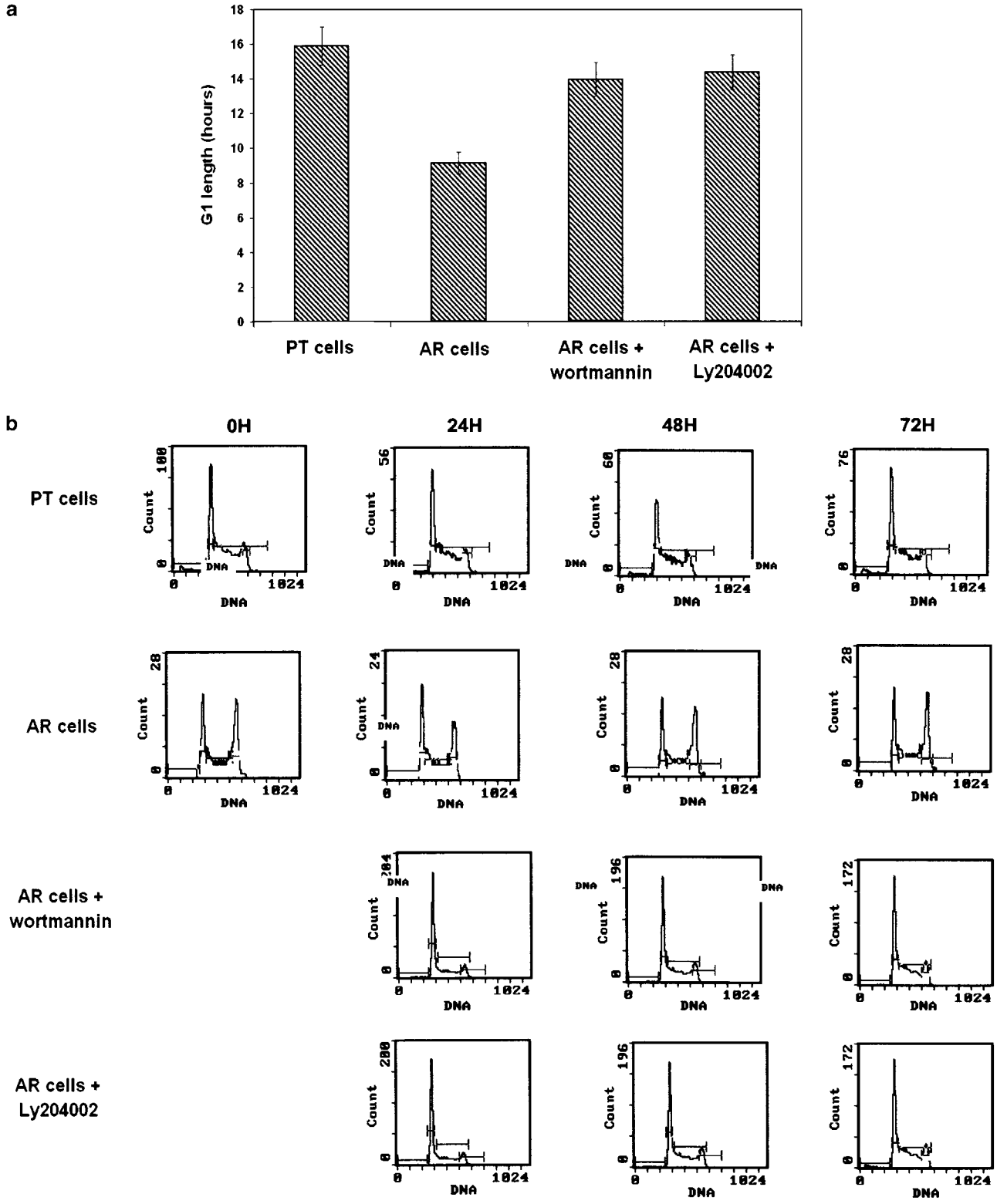

Figure 5 Estimation of G1 length in PT, AR, and inhibitor-exposed AR cells. Results are the mean ± s.d. of three different experiments (a). (b) Representative histograms showing flow-cytometric analysis of PI-stained samples.

This indicated that some Akt targets may reside within this organelle. Also for this reason, we focused our attention on forkhead transcription factors which, when they are not phosphorylated by Akt, localize to the nucleus[9–11,13] where they negatively regulate cell proliferation and survival.[27–29] We detected no differences between HL60PT and AR cells as far as the overall expression of both FoxO1 and FoxO3 was concerned. However, FoxO1 and FoxO3 were hyperphos-

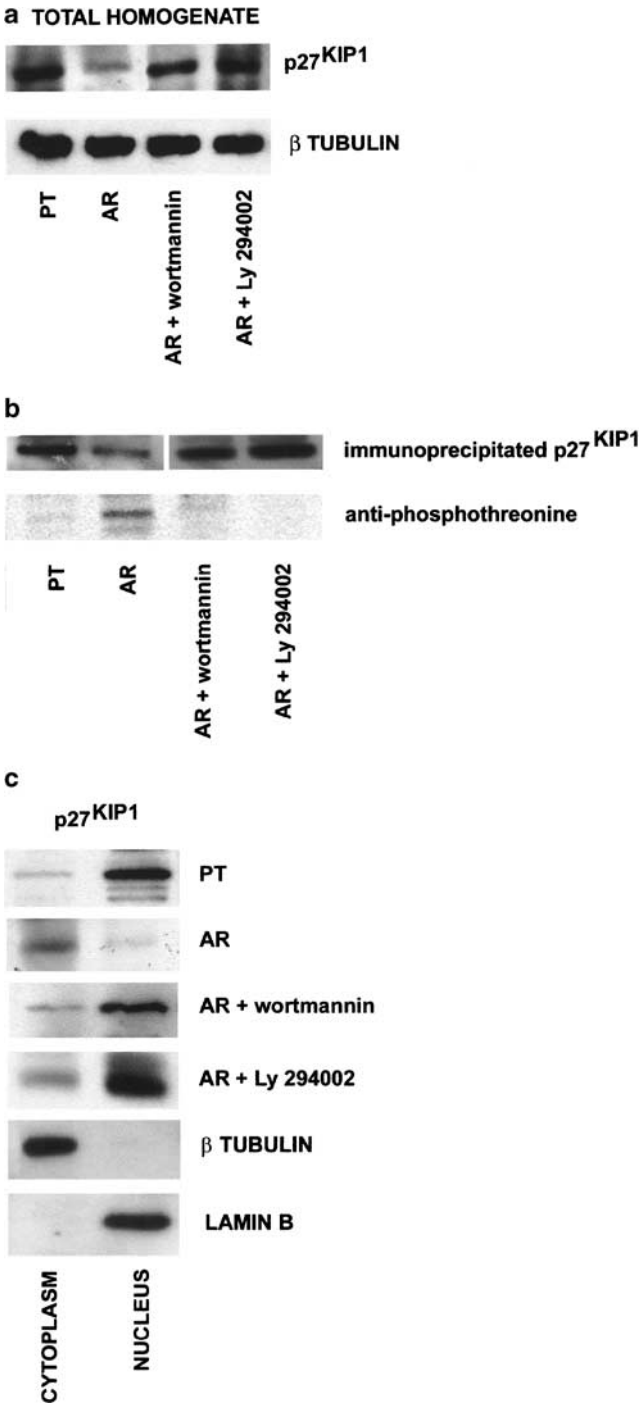

Figure 6 The expression, threonine phosphorylation, and localization of $p27^{Kip1}$ are regulated through the PI3K/Akt pathway. (a) Western blot analysis for the expression levels of $p27^{Kip1}$ in HL60 PT and AR cells, and in AR cells treated with wortmannin or Ly294002. Blots were also probed with antibody to β tubulin to ensure equal gel loading. (b) Cells were lysed and immunoprecipitated with an antibody to $p27^{Kip1}$. The immunoprecipitates were probed with antibodies to either $p27^{Kip1}$ or phosphorylated threonine residues. (c) Western blot analysis showing subcellular distribution of $p27^{Kip1}$ in HL60 PT and AR cells, and in AR cells treated with wortmannin or Ly294002. In both (a) and (c), 80 μg of protein, run on SDS-PAGE, was blotted to each lane. The purity of the fractions was evaluated by immunochemical analysis with antibody to β tubulin and lamin B. The blots are representative of three different experiments. In all the experiments, wortmannin (0.1 μM) or Ly294002 (10 μM) was present for 24 h before harvesting.

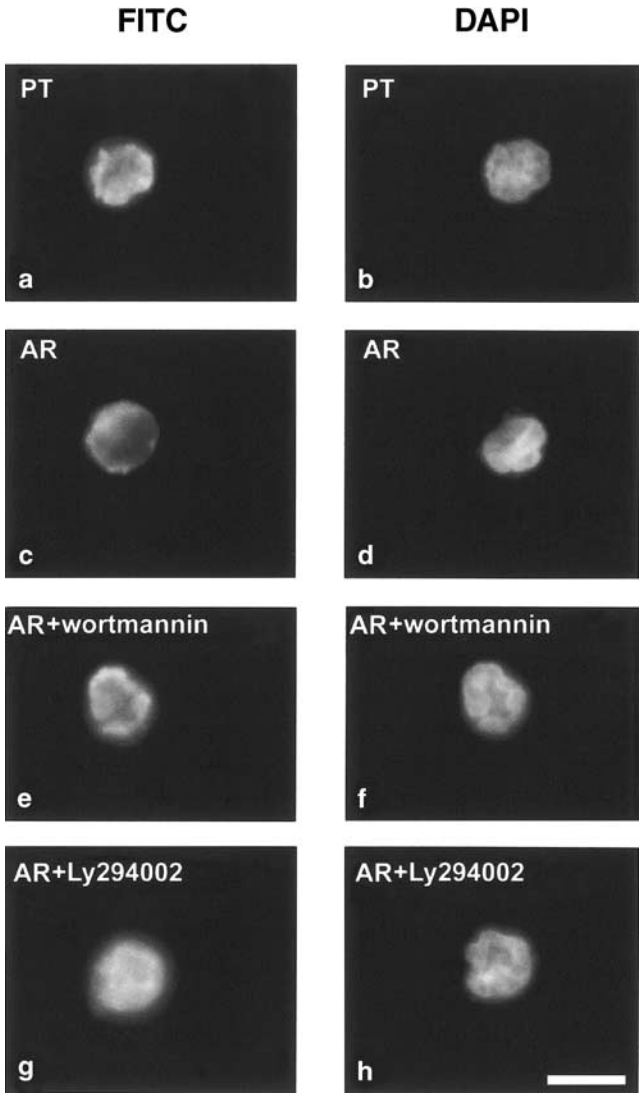

Figure 7 The subcellular localization of $p27^{Kip1}$ is under the control of the PI3K/Akt axis. Immunofluorescence staining showing subcellular localization of $p27^{Kip1}$ in HL60 PT and AR cells, and in AR cells treated with wortmannin or Ly294002. Cells adherent to coverslips were fixed, permeabilized and stained with a monoclonal antibody to $p27^{Kip1}$, which was then revealed by a FITC-conjugated anti-mouse IgG. Samples were counterstained for DNA by means of DAPI. Wortmannin (0.1 μM) or Ly294002 (10 μM) was present for 24 h prior to fixation. Scale bar: 5 μM.

phorylated on Akt-targeted sites in HL60AR cells and the phosphorylation levels were downregulated by two unrelated pharmacological inhibitors of PI3K. Moreover, cell fractionation experiments revealed a predominantly cytoplasmic localization of FoxO1 and FoxO3 in AR cells, consistently with an Akt-mediated phosphorylation. Taken together, these results suggested that FoxO1 and FoxO3 are downstream targets of PI3K/Akt signaling also in HL60 leukemia cells.

In mammalian cells, proliferation is under the control of factors that regulate the transition between cell cycle stages at two main check points.[26] The first one is at the G1/S phase transition for initiation and completion of DNA replication in S phase.[30,31] The second checkpoint is at the G2/M phase transition and controls mitosis and cell division.[32] When

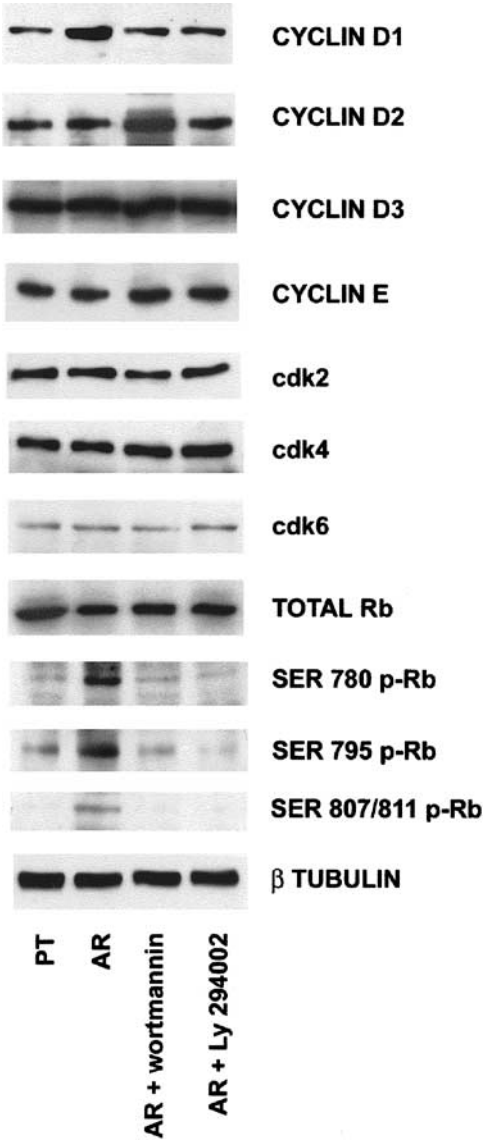

Figure 8 The PI3K/Akt axis controls cyclin D1 expression and phosphorylation of 110 kDa Rb protein. Western blot analysis for G1 cyclins (cyclin D1, D2, D3, E), cdks, total Rb and p-Rb forms in HL60 PT and AR cells, and in AR cells treated with wortmannin or Ly294002. Blots were also probed with antibody to β tubulin to ensure equal gel loading (80 μg/lane). Immunoreactive bands were revealed by ECL. In all the experiments, wortmannin (0.1 μM) or Ly294002 (10 μM) was present for 24 h before harvesting. All the blots are representative of three separate experiments.

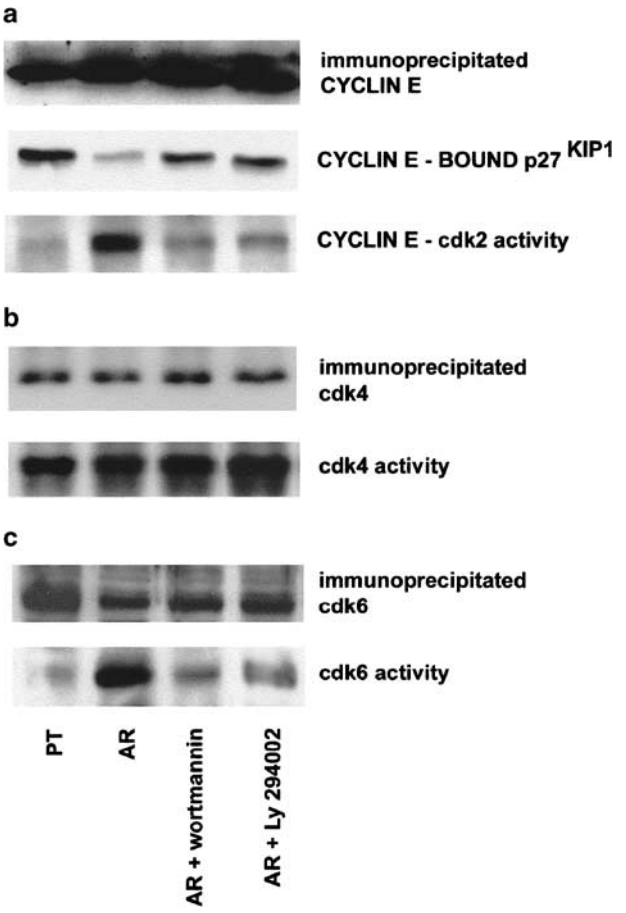

Figure 9 The activity of cdk2 and cdk6 is under the control of the PI3K/Akt axis. (a) cyclin E was immunoprecipitated from lysed cells by a specific polyclonal antibody, then the blots were probed with antibody to either cyclin E or $p27^{Kip1}$; cyclin E-associated ckd2 activity was assayed on immunoprecipitates *in vitro* using histone H1 as substrate. (b) cdk4 was immunoprecipitated from lysed cells by a specific polyclonal antibody and its activity was determined *in vitro* using GST-Rb as a substrate in an immunocomplex assay. (c) cdk6 was immunoprecipitated from lysed cells by a specific polyclonal antibody and its activity was determined *in vitro* using GST-Rb as a phosphorylation substrate. (a), (b), and (c) are representative of three separate experiments.

compared with PT cells, HL60AR cells displayed a marked increase in the amount of cells in either the S or G2/M phase of the cell cycle, as evidenced by flow cytometric analysis. Treatment with either wortmannin or Ly294002 decreased the number of HL60AR cells in both S and G2/M phase and increased those in G1. Furthermore, AR cells had a G1 transit type considerably shorter than PT cells, and the length of their G1 phase could be lengthened by wortmannin or Ly294002. Therefore, HL60AR cells present a double advantage over HL60PT cells because they are much less sensitive to apoptotic stimuli[16,17] and they proliferate faster.

Since recent results have shown that FoxO3 may negatively control the expression of $p27^{Kip1}$,[14] a well-established regulator of the G1/S phase transition, we analyzed its expression by Western blot. $p27^{Kip1}$ is a direct inhibitor of cdk2, one of the cdks responsible for the activation of E2F1 transcription factors that promote DNA replication.[33] We have found that $p27^{Kip1}$ levels are lower in HL60AR cells when compared with PT cells. Also, the expression of $p27^{Kip1}$was sensitive to wortmannin and Ly294002, indicating an involvement of the PI3K/Akt axis in the control of $p27^{Kip1}$ protein levels. However, the decreased expression of $p27^{Kip1}$ is not the only factor that could explain the observed differences between HL60 PT and AR cells as far as cell cycle progression was concerned. Indeed, we have also found that in HL60AR cells $p27^{Kip1}$ is mainly localized in the cytoplasm, whereas in PT cells it is predominantly located in the nucleus. Furthermore, in HL60 cells $p27^{Kip1}$ is hyperphosphorylated on threonine residues and this phosphorylation was inhibited by either wortmannin or Ly294002. These results strongly suggested that $p27^{Kip1}$ is a direct target of the PI3K/Akt axis in HL60 cells in agreement with recent finding by others, obtained in breast tumors.[24,25,34] Indeed, it has been shown that

Akt phosphorylates $p27^{Kip1}$ both *in vitro* and *in vivo* and phosphorylation occurs on a threonine residue (Thr 157) within the nuclear localization signal (NLS) of $p27^{Kip1}$,[34] so that nuclear import of $p27^{Kip1}$ is impaired. Viglietto *et al*[24] were able to generate an antibody that specifically recognizes the Akt-phosphorylated Thr 157 of $p27^{Kip1}$. Even though we could not use an antibody specific for Thr 157-phosphorylated $p27^{Kip1}$, our results, obtained with an antibody raised to phosphorylated threonine residues, strongly suggested that, also in HL60AR cells, $p27^{Kip1}$ is a target of the PI3K/Akt pathway and this results in its relocation from nucleus to cytoplasm. So far, a shifted location of $p27^{Kip1}$ from nucleus to cytoplasm has been observed in carcinomas of breast, thyroid, esophagus, and colon,[35] but our findings point to the likelihood that it may be a feature of other types of neoplasia.

Nevertheless, there is yet another reason that may explain why HL60AR cells have a cell cycle progression faster than PT cells. Indeed, they express higher levels of cyclin D1 and, as a consequence, a higher level of p110 p-Rb protein. In contrast, the levels of cyclin D2, D3, and E, as well as those of cdk2, cdk4, and cdk6 were similar in PT and AR cells. Enhanced levels of cyclin D1 and hyperphosphorylation of p110 Rb protein detected in AR cells were sensitive to wortmannin or Ly294002, an observation which indicates their dependency on the PI3K/Akt axis. Both cyclin D in complex with cdk4 or cdk6 and cyclin E-cdk2 phosphorylate p110 Rb protein, a well-known tumor suppressor. The tumor suppressor activity of Rb protein relies on gating S phase entry through its ability to repress genes activated by the E2F family of transcription factors.[36] In fact, p-Rb releases members of the E2F family that play an integral role in cell cycle progression by inducing the expression of genes required for S phase entry.

Consistently with a downregulation and cytoplasmic relocalization of $p27^{Kip1}$, AR cells had higher levels of cyclin E-bound cdk2 activity. They also showed enhanced ckd6 activity, presumably due to upregulation of cyclin D1, whereas cdk4 activity was the same in either PT or AR cells and was not sensitive to treatment of AR cells with PI3 K inhibitors. Previous results have indicated that the levels of $p27^{Kip1}$ were decreased, whereas those of cyclin D1, D2, D3, and E were elevated, in HL60 cells resistant to 1,25-dihydroxyvitamin D3 when compared with sensitive cells.[37,38] Accordingly, our HL60AR cells are resistant to ATRA[16] and displayed lower levels of $p27^{Kip1}$ and a higher amount of cyclin D1 when compared with ATRA-sensitive (PT) cells.

Previous findings from our laboratory have highlighted the importance of the NF-κB transcription factor in determining HL60AR cell resistance to TRAIL,[17] while those reported in this paper have underscored the key role for forkhead transcription factors in regulating cell cycle progression in the same cell clone. As far as a possible CREB activation was concerned, our unpublished findings have revealed that the activity of this PI3K/Akt-dependent transcription factor is not upregulated in HL60AR cells.

So far, the PI3K/Akt pathway has been scarcely investigated in patients suffering from acute leukemia. It should be considered, however, that there are some cases of acute myeloid leukemia in which the PTEN tumor suppressor is absent or nonfunctional.[39–41] Since PTEN is a fundamental negative regulator of Akt activity,[42] it might be that in these leukemias the Akt pathway is upregulated with a consequent resistance to multiple apoptotic stimuli and high proliferation rates. Moreover, very recent evidence has shown the occurrence of a possible dominant-negative mutation of the SHIP gene in acute myeloid leukaemia.[43] SHIP is a hematopoietic-specific inhibitory phosphatase[44] which dephosphorylates phosphatidylinositol $(3,4,5)P_3$ to phosphatidylinositol $(3,4)P_2$, thus negatively influencing Akt activity.

Overall, our findings are in agreement with those of other laboratories that have recently demonstrated the importance of the PI3K/Akt/FoxO factor pathway in controlling the proliferation of both human and murine hematopoietic cells lines[14,45,46] as well as of human freshly isolated T cells[14] or $CD34^+$ hematopoietic progenitors cells treated with erythropoietin.[47] Intriguingly, it has also been shown that FoxO1 is a downstream effector of STI 571-induced cell cycle arrest in the KCL22 cell line established from the bone marrow of a patient with blastic crisis of bcr-abl-positive chronic myelogenous leukemia.[48] Consistently, exposure of these cells to STI 571 led to overexpression of $p27^{Kip1}$. Therefore, we may infer that our findings are not restricted to a single cell line.

In light of this, in the future, pharmacological inhibitors of PI3K/Akt axis might prove very valuable for slowing down the growth rate of certain types of human leukemia other than for sensitizing them to therapeutic agents.

Acknowledgements

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