THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 18, Issue of May 6, pp. 18120–18129, 2005
Printed in U.S.A.

$p21^{Cip1}$ and $p27^{Kip1}$ Induce Distinct Cell Cycle Effects and Differentiation Programs in Myeloid Leukemia Cells*

Received for publication, January 21, 2005, and in revised form, February 23, 2005
Published, JBC Papers in Press, March 3, 2005, DOI 10.1074/jbc.M500758200

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The cyclin-dependent kinase (Cdk) inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ have been proposed to exert redundant functions in cell cycle progression and differentiation programs, although nonoverlapping functions have also been described. To gain further insights into the relevant mechanisms and to detect possible functional differences between both proteins, we conditionally expressed $p21^{Cip1}$ and $p27^{Kip1}$ in K562, a multipotent human leukemia cell line. Temporal ectopic expression of either $p21^{Cip1}$ or $p27^{Kip1}$ arrested proliferation, inhibited Cdk2 and Cdk4 activities, and suppressed retinoblastoma phosphorylation. However, whereas $p21^{Cip1}$ arrested cells in both G_1 and G_2 cell cycle phases, $p27^{Kip1}$ blocked the G_1/S-phase transition. Furthermore, although both $p21^{Cip1}$ and $p27^{Kip1}$ associated with Cdk6, only $p27^{Kip1}$ significantly inhibited its activity. Most importantly, each protein promoted differentiation along a distinct pathway; $p21^{Cip1}$ triggered megakaryocytic maturation, whereas $p27^{Kip1}$ resulted in the expression of erythroid markers. Consistently, $p21^{Cip1}$ and $p27^{Kip1}$ were rapid and transiently up-regulated when K562 cells are differentiated into megakaryocytic and erythroid lineages, respectively. These findings demonstrate distinct functions of $p21^{Cip1}$ and $p27^{Kip1}$ in cell cycle regulation and differentiation and indicate that these two highly related proteins possess unique biological activities and are not functionally interchangeable.

$p21^{Cip1}$ (hereafter termed p21) and $p27^{Kip1}$ (hereafter termed p27) belong to the Cip/Kip family of cyclin-dependent kinase (Cdk)[1] inhibitors (CKIs) and have been extensively characterized as negative regulators of the G_1-phase cell cycle progression. p21 and p27 are found in complexes composed of Cdks and their cyclin-activating partners and inhibit their catalytic activities. These interactions are mediated by the conserved amino-terminal portions of the proteins, which enable them to bind both the cyclin and Cdk subunits. Cip/Kip proteins can bind and inhibit a broad spectrum of Cdks, with the strongest inhibitory activity manifested against Cdk2 complexes (1, 2). Most intriguingly, Cip/Kip CKIs have also been reported as constituents of active cyclin D-Cdk4/6 complexes, perhaps promoting their assembly (3–6), although other groups have presented conflicting results (7, 8).

Several lines of evidence suggest that p21 and p27 exert similar effects on cyclin-Cdk complexes and cell cycle progression as follows: (*a*) both proteins have conserved sequences in their inhibitory domains; (*b*) both proteins mediate the inhibition of Cdk2 complexes by a wide range of antimitogenic signals, and (*c*) the stability of cyclin D-Cdk complexes depends redundantly upon p21 and p27 (6). However, other observations indicate that p21 and p27 also have nonoverlapping functions and are not biologically equivalent. (i) p21 and p27 are often differentially expressed; for example, in fibroblasts p27 levels are high in quiescent cells, and p27 destruction is required for the G_1/S traverser, whereas p21 levels start low and typically increase during cell cycle entry (1). (ii) The efficiency of Cdk4 inhibition *in vitro* has been reported to be different for p21 and p27 (4). (iii) p21 and p27 knock-out mice display different phenotypes (9–11).

Most of the available information on the activities of p21 and p27 concerns their role on cell cycle control and using fibroblasts as the predominant model system. However, p21 and p27 have also been implicated in multiple biological functions unrelated to cell cycle, such as apoptosis, cell motility, and differentiation (12, 13). During the hematopoietic differentiation program, both CKIs are up-regulated by multiple agents that trigger myeloid, megakaryocytic, and erythroid lineages (reviewed in Refs. 14 and 15). Overexpression of either p21 or p27 in cell lines induces monocytic (16–19) or megakaryocytic differentiation (20, 21), whereas suppression of these CKIs by antisense techniques decreases myeloid differentiation (22–25). In general, these observations support the notion that p21 and p27 exert similar functions in differentiation. However, prior studies have been performed with cell lines capable of differentiation only toward a single lineage, thus pre-conditioning their response to modulation of p21 and p27 expression. Indeed, experiments performed with hematopoietic cells from knock-out mice suggest unique functions as follows: p21 promotes progenitor cell replication (26), whereas p27 restrains progenitor cell expansion (27). Furthermore, normal hematopoietic cells show sequential peaks of p21 and p27 expression

* This work was supported in part by Grant SAF02-4193 from the Spanish Ministerio de Ciencia y Tecnología (to J. L.), a grant from the Fondo de Investigaciones Sanitarias (F.I.S.) (to M. D. D.), and Grant GM41690 from the National Institutes of Health (to J. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by Fundación Marqués de Valdecilla, F.I.S., and Fundación Científica de la Asociación Española Contra el Cáncer.

¶ Supported by a fellowship of the Ministerio de Educación y Ciencia, Spain.

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[1] The abbreviations used are: Cdk, cyclin-dependent kinase; ARA-C, 1-β-arabinofuranosylcytosine; BrdUrd, bromodeoxyuridine; CKI, Cdk inhibitory protein; DAPI, 4′-6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; Rb, retinoblastoma protein.

during progression to terminal differentiation (28, 29), indicating that they may be required at specific stages.

In this study, we have investigated the possible differential roles of p21 and p27 in the regulation of cell cycle progression and differentiation using the K562 cell line as a model system. K562 human leukemia cells represent a useful tool to address this question because (i) K562 cells can mature along distinct hematopoietic lineages (30, 31), and (ii) they are deficient in the CKIs $p15^{INK4b}$ and $p16^{INK4a}$ (32), which simplifies the analysis of the effects of p21 and p27. Using K562 derivatives with conditional expression of either p21 or p27, we show that whereas the up-regulation of both p21 and p27 at comparable levels provokes growth arrest, p27 strongly inhibits Cdk6 complexes and p21 does not. Moreover, there is a striking difference in the biological effects elicited, as p21 directs cells toward megakaryocytic differentiation and p27 provokes an erythroid differentiation response.

EXPERIMENTAL PROCEDURES

Plasmids—cDNAs encoding human p21 and p27 (M. Serrano, Centro Nacional de Investigaciones Oncológicas, Madrid, Spain) were subcloned into the pMT-CB6+ (neo^r) vector, which contains the sheep metallothionein promoter for Zn^{2+}-inducible expression (F. J. Rausher, Wistar Institute, Philadelphia), to generate plasmids pMTp21 and pMTp27. To construct pCEFLp27AS, p27 cDNA was inserted in the antisense orientation into the pCEFL vector, where its expression is directed by the EF-1α promoter.

Cell Culture Procedures—Cell line K562 (from ATCC) was derived from a human chronic myeloid leukemia in blast crisis. Kp21, Kp27, and KMT sublines were obtained by electroporation of K562 cells with plasmids pMTp21, pMTp27, and pMT-CB6+, respectively. Individual clones were selected with 0.5 mg/ml G418. To generate the Kp21-p27AS subline (Kp21 cells expressing p27-antisense), Kp21 clone 4 was cotransfected with pCEFLp27AS and pLPCX, which contains a puromycin resistance gene, and cells were selected with 0.5 mg/ml G418 and 1 μg/ml puromycin. Kp53C1, a K562-derived cell line expressing the thermosensitive $p53^{Val\text{-}135}$ mutant, has been described (33). Cells were grown in RPMI 1640 medium (PerkinElmer Life Sciences) supplemented with 10% fetal calf serum and gentamycin (80 μg/ml). When indicated, cells (2.5×10^5 cells/ml) were treated with 75 μM $ZnSO_4$ to induce p21 or p27 expression and 100 nM staurosporine (Sigma) to induce megakaryocytic differentiation (30) or 1 μM cytosine arabinoside (ARA-C; Sigma) (31) to induce erythroid differentiation. Morphological differentiation was monitored by examination of May-Grünwald-Giemsa stained cells using established cytological criteria. Hemoglobin-producing cells were scored by benzidine staining (31). At least 200 cells were counted in each determination.

Flow Cytometric Analysis—Cells were pulsed with 30 μM bromodeoxyuridine (BrdUrd; Roche Applied Science) for 1 h prior to harvest. The cells were then collected by centrifugation, fixed in 90% ethanol at 4 °C, washed with PBS, and incubated at 37 °C for 30 min with RNase (200 μg/ml). Cells were then incubated for 30 min with 1 N HCl, washed once with 0.1 M boric buffer, pH 8.3, and once with PBSTXB (PBS 0.1%, Triton X-100 and 0.5% bovine serum albumin). Cells were incubated with an FITC-conjugated anti-BrdUrd monoclonal antibody for 30 min (Roche Applied Science, diluted 1:40 in PBSTXB), washed in PBSTXB, and resuspended with 10 μg/ml propidium iodide (Sigma). The stained cells were analyzed by flow cytometry on a FACScan flow cytometer (BD Biosciences).

RNA Analysis—Total RNA was isolated using the RNeasy kit (Qiagen). Northern blots were hybridized with ^{32}P-labeled probes as described (30). The probes were fragments of human p21 and p27 cDNAs, ϵ-globin cDNA (34), and integrin β_3 cDNA (Angel Corbi, Centro de Investigaciones Biológicas, Madrid, Spain).

Real Time PCR Analysis—First-strand cDNA was synthesized from 1 μg of total RNA using SuperScript™ II RNase reverse transcriptase (Invitrogen) using random primers. For real time PCR, the QuantiTect™ SYBR green PCR kit (Qiagen) was used. Primers for cDNAs of human c-Mpl, glycophorin A, ribosomal protein S14, and β_1-tubulin (corresponding to the isoform-specific 5′-untranslated region) are available upon request. For each gene, duplicate PCRs were performed for protein S14 and the test gene on the same 96-well plate on an ICycler iQ™ apparatus (Bio-Rad).

Immunoblotting—Immunoblots were performed as described previously (33). Densitometric analyses were performed using a ChemiDoc (Bio-Rad) instrument. The antibodies were from the following suppliers: anti-p21 (C-19), anti-p27 (C-19), anti-Cdk2 (M2), anti-Cdk4 (C-22), anti-Cdk6 (C-21), and anti-Rb (C-15) were from Santa Cruz Biotechnology; anti-cyclin D (06-137) was from Upstate Biotechnology, Inc.; anti-α-tubulin was a gift from N. Cowan (New York University, New York). Recombinant p21 (p21-glutathione *S*-transferase-tagged fusion protein, 48 kDa) and p27 (p27-polyhistidine-tagged fusion, 28 kDa) were from Santa Cruz Biotechnology.

Immunoprecipitation and Kinase Assays—Cdk2, Cdk4, Cdk6, and cyclin D1-associated kinase activities assay were performed as described previously (35). Protein extracts (500 μg per assay) were immunoprecipitated with 1 μg of the following antibodies: anti-Cdk2 (M2), anti-Cdk6 (C-21, Santa Cruz Biotechnology), anti-Cdk4 (Ab-1), and anti-cyclin D1 (Ab-2, Neomarkers), and collected on Gammabind-Sepharose beads (Amersham Biosciences). After extensive washing, kinase activity was assayed using histone H1 for Cdk2 immunoprecipitates or Rb for Cdk4, Cdk6, and cyclin D1 immunoprecipitates. The kinase reaction contained 50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 2.5 mM EGTA, 1 mM dithiothreitol, 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM NaF, 50 μM cold ATP, 10 μCi of [γ-^{32}P]ATP (6000 Ci/mmol; Amersham Biosciences), and 5 μg of histone H1 (Roche Applied Science) or 2 μg of Rb (Upstate Biotechnology, Inc.). The reactions were incubated at 30 °C for 30 min. The phosphorylated proteins were separated by SDS-PAGE, transferred to Immobilon P membranes (Millipore), and quantified using a PhosphorImager (Bio-Rad). The membranes were subsequently used for immunoblot analysis. For p27 and p21 immunoprecipitation, 500 μg of protein extract was incubated with 1 μg of anti-p27 (C-19) or anti-p21 (C-19, Santa Cruz Biotechnology) antibodies, and the immunoprecipitated material was collected on protein A-agarose beads.

Immunofluorescence Staining—Cytospin preparations were fixed in 3.7% paraformaldehyde/PBS for 15 min and permeabilized with PBS, 0.5% Triton X-100 for 30 min. Slides were incubated with a 1:100 dilution of anti-p21(C-19) or anti-p27 (C-19, Santa Cruz Biotechnology) antibodies. Anti-rabbit FITC, diluted 1:100, was used as the secondary antibody (Dako). Samples were mounted with Vectashield (Vector Laboratories) containing 4′,6-diamidino-2-phenylindole (DAPI) to stain nuclei and examined under a fluorescence microscope (Zeiss Axioskop).

RESULTS

Inducible Expression of p21 and p27—In order to compare the effects of ectopic expression of p21 and p27 on cell cycle progression and differentiation of K562 cells, we generated stable sublines with conditional expression of transfected cDNAs (designated Kp21 and Kp27, respectively). As a control, K562 cells were transfected with the empty pMT-CB6+ vector (KMT cells). Several Kp21 clones showed $ZnSO_4$-inducible p21 protein expression (Fig. 1*A*). p21 levels were not supra-physiological but equivalent to those of endogenous p21 induced by p53 activation in K562 cells (33). Fig. 1, *B* and *C,* shows the kinetics of p21 induction of one representative clone (Kp21-4), which was chosen for most of the additional experiments. $ZnSO_4$ elicited a rapid increase of p21 mRNA (Fig. 1*B*) and protein (Fig. 1*C*) within 3 h, peaking between 6 and 12 h, and returning to base line after 2 days. The decrease in p21 expression (and p27, see below) is because of transient activity of metallothionein promoter in the presence of Zn^{2+} and has been already observed for several genes in K562 (34, 36). Because p21 has different functions depending on the cell compartment where the protein is expressed (13), we performed immunofluorescence analysis of Kp21-4 cells. As shown in Fig. 1*D*, strong nuclear staining of p21 was observed in Zn^{2+}-treated Kp21-4 cells.

Similar analyses were performed with Kp27 cells. Multiple transfectants conditionally expressed exogenous p27 mRNA after Zn^{2+} addition (Fig. 2*A*). Kinetics of p27 mRNA expression in one representative clone (Kp27-5) is shown in Fig. 2*B*. p27 mRNA levels increased within 2 h of Zn^{2+} addition and returned to base-line levels after 24 h. Levels of p27 protein peaked at 24 h, declined gradually thereafter, and were still detectable at 3 days after Zn^{2+} treatment (Fig. 2*C*). A strong p27 signal was observed by immunofluorescence in Kp27 cells

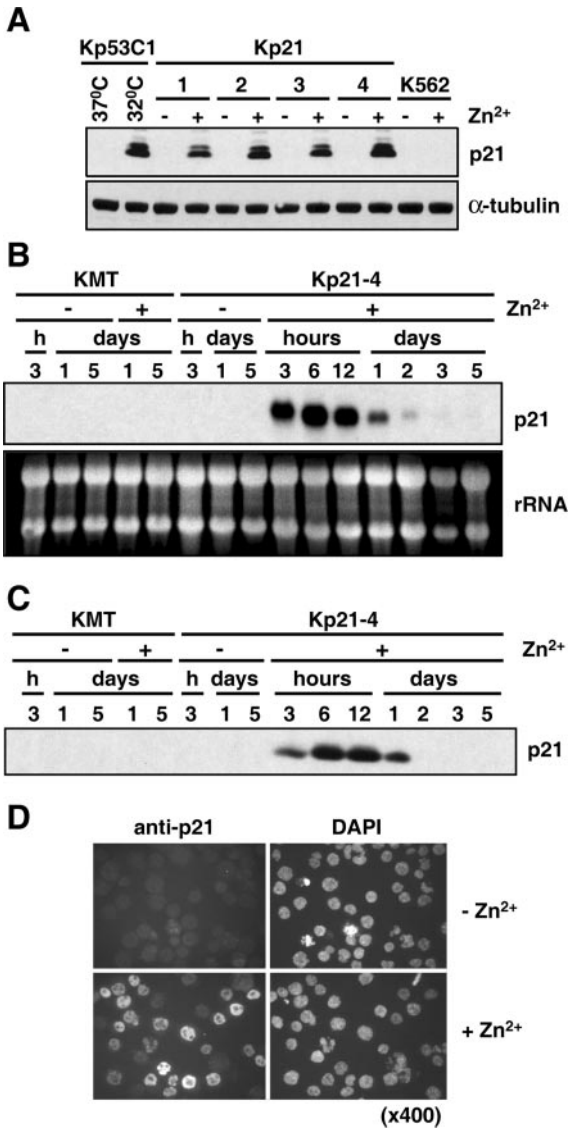

FIG. 1. **Characterization of K562 sublines with conditional p21 expression.** *A*, immunoblot analysis of p21 expression in p21-transfected K562 sublines (Kp21) after Zn^{2+} treatment, and in a $p53^{Val-135}$-transfected K562 subline (Kp53C1) (33) after temperature shift to 32 °C to activate p53. Kp21 clones and parental cells (K562) were incubated for 16 h in medium with (+) or without (−) 75 μM $ZnSO_4$. Kp53C1 cells were cultured for 12 h at 32 °C to activate p53 or continuously at 37 °C. Equivalent loading was assessed with an anti-α-tubulin antibody. *B*, Northern hybridization analysis of p21 mRNA induction by Zn^{2+} in Kp21 clone 4 (*Kp21-4*) and in K562 cells transfected with the empty vector (KMT cell line). Loading was assessed by ethidium bromide staining of rRNAs in the filter (*lower panel*). *C*, kinetics of p21 protein induction in Kp21-4 and KMT cells in response to Zn^{2+} treatment assessed by immunoblot analysis. *D*, immunofluorescent analysis of the subcellular localization of ectopically expressed p21. Kp21-4 cells were cultured in the presence or absence of Zn^{2+} for 24 h and stained with an anti-p21 antibody. *Right panels* show nuclei counter-stained with DAPI.

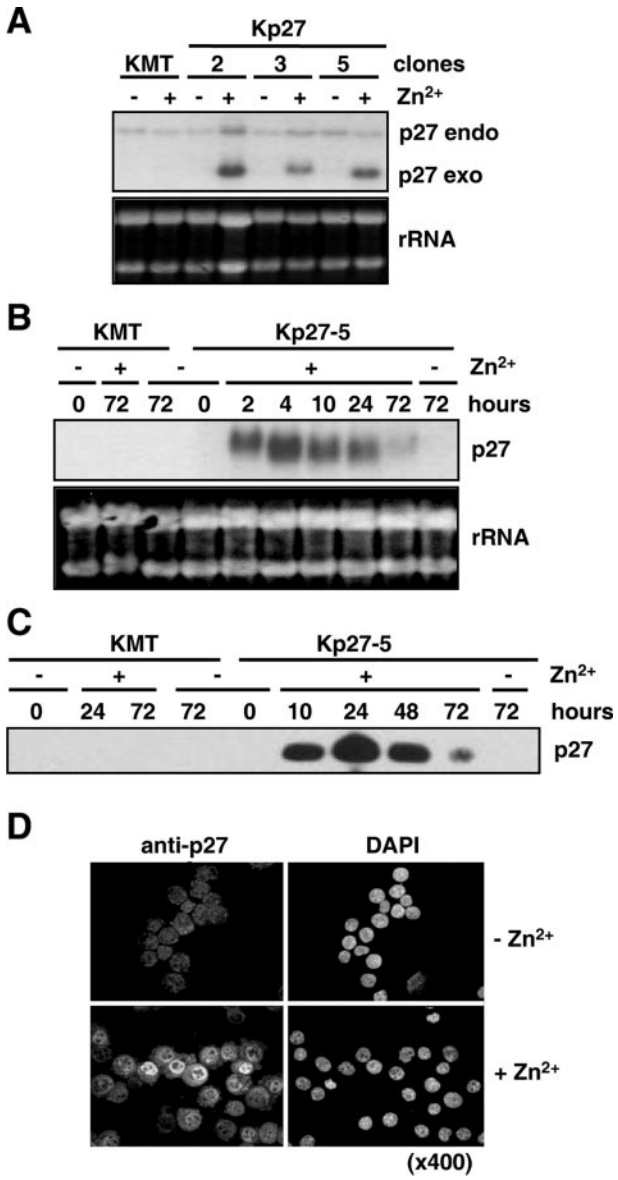

FIG. 2. **Characterization of K562 sublines with conditional p27 expression.** *A*, Northern hybridization analysis of p27 mRNA expression in p27-transfected K562 sublines (Kp27) after Zn^{2+} treatment. Kp27 clones (*clones 2–5*) and control KMT cells were incubated for 24 h in medium with (+) or without (−) 75 μM $ZnSO_4$. Loading was assessed by ethidium bromide staining of rRNAs in the filter (*lower panel*). *B*, time course of p27 mRNA induction by Zn^{2+} in Kp27 clone 5 (*Kp27-5*). *C*, kinetics of p27 protein induction in Kp27-5 and KMT cells in response to Zn^{2+} treatment assessed by immunoblot analysis. *D*, immunofluorescent analysis of the subcellular localization of ectopically expressed p27. Kp27-5 cells were cultured in the presence or absence of Zn^{2+} for 24 h and stained with an anti-p27 antibody. *Right panels* show nuclei counter-stained with DAPI.

cultured in the presence of Zn^{2+} for 1 day (Fig. 2*D*). Although some p27 could be detected in the cytoplasm, most of the protein localized to the nuclei.

Induction of p21 or p27 Leads to Growth Arrest—Exposure of vector-transfected KMT (Fig. 3*A*) or parental K562 cells (data not shown) to 75 μM $ZnSO_4$ did not affect their proliferation. In contrast, treatment of Kp21-4 or Kp27-5 cells with Zn^{2+} resulted in almost complete inhibition of proliferation (Fig. 3*A*). Similar results were observed in other p21- and p27-transfected clones (data not shown). p21 elicited an irreversible arrest because proliferation was not restored by the removal of Zn^{2+} after 6 h of treatment; in contrast, the effects of p27 were reversible (data not shown).

Cells were pulsed with BrdUrd and subsequently stained with anti-BrdUrd antibodies and propidium iodide to determine the active S-phase fraction and cell cycle distribution, respectively. Flow cytometric analysis showed no significant changes in control KMT cells in the absence or presence of Zn^{2+} (Fig. 3*B*). However, propidium iodide staining of Zn^{2+}-treated Kp21-4 cells showed a significant reduction in the S-phase fraction and an accumulation of cells in the G_2-phase, exhibiting also a G_1 blockade. In agreement, numbers of BrdUrd-positive cells were also markedly reduced. Similarly, Zn^{2+}-treated Kp27-5 cells exhibited a strong reduction in BrdUrd

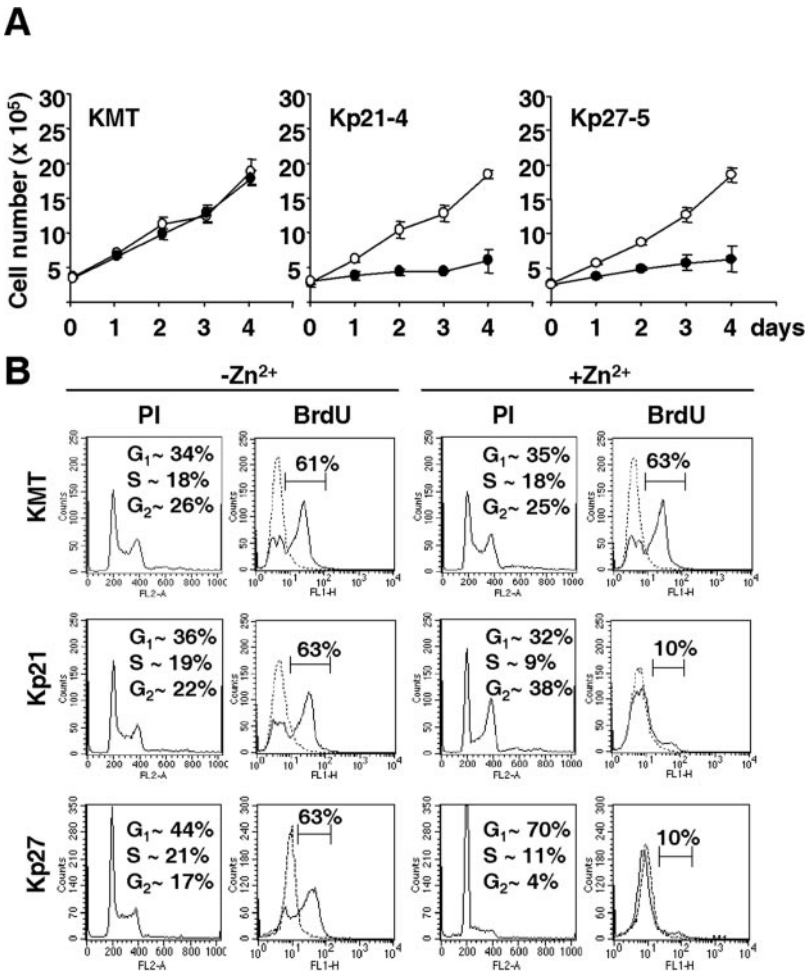

FIG. 3. **Effect of ectopic p21 and p27 expression on proliferation of K562 cells.** *A*, growth curves of KMT, Kp21-4, and Kp27-5 cultures. Cells were inoculated at 2.5×10^5 cells/ml, cultured in the presence (●) or absence (○) of Zn^{2+}, and counted daily with a hemocytometer. Mean values ± S.E. ($n = 3$) are shown. *B*, two-parameter flow cytometric analysis of cell cycle progression. Cultures in exponential phase of growth were treated with Zn^{2+} for 14 h for KMT and Kp21-4 cells and for 24 h for Kp27-5 cells (conditions were chosen to elicit maximal p21 and p27 protein expression). *Left panel*, DNA content histograms analyzed by propidium iodide (*PI*) staining, showing the percentages of cells in G_1-, S-, and G_2/M-phases. *Right panel*, BrdUrd (*BrdU*) incorporation analyzed after staining with anti-BrdUrd-FITC antibody, showing the fraction of cells in active S-phase. As control, cells cultured in the absence of BrdUrd and incubated with antibody were also analyzed (*dotted line*).

incorporation; however, in contrast to Kp21-4 cells, propidium iodide staining of Kp27-5 cells showed an almost exclusive accumulation in G_0/G_1-phase (Fig. 3*B*). Expression of neither p21 nor p27 induced apoptosis, as assessed by the absence of a sub-G_1 population or by morphological analysis of Giemsa-stained cells (data not shown).

p21 and p27 Exert Differential Effects on Cdk6 Activity—To gain insight into the mechanisms by which these CKIs cause cell cycle arrest, we analyzed the activity of the G_1 Cdks. Cdk2 activity was strongly and rapidly inhibited in Kp21-4 cells after Zn^{2+} addition (Fig. 4*A*). The loss of activity was not accompanied by decreased Cdk2 protein expression (Fig. 4*C*), suggesting that the kinase was inhibited by p21 binding. Indeed, p21 was detected in Cdk2 immunoprecipitates (Fig. 4*A*). We also tested the effect of p21 induction on Cdk4 and Cdk6 activity, and on the Rb kinase activity of cyclin D1 immunoprecipitates. Cdk4 activity was not significantly altered after 6 h of p21 induction, but it was clearly reduced after 24 h (Fig. 4*A*, compare kinase activities with Cdk4 levels in the immunoprecipitates). p21 protein was detected in Cdk4 immunoprecipitates at both times (Fig. 4*A*). p21 protein was also detected in Cdk6 immunoprecipitates, but strikingly, no significant effect on Cdk6 activity was detected at any time point. Fig. 4*B* shows a histogram of the kinase activity of Cdk2, Cdk4, and Cdk6 normalized to the levels of each immunoprecipitated Cdk. The results (corresponding to three independent experiments) confirm the lack of inhibition of Cdk6 by p21 and that Cdk2 is more potently inhibited than Cdk4. In cyclin D1 immunoprecipitates, only a modest reduction in Rb kinase activity (10%) was observed after 24 h of Zn^{2+} treatment (Fig. 4*A*). p21 induction did not affect overall the Cdk4 or Cdk6 protein levels (Fig. 4*C*). To confirm that p21 was indeed present in these kinase complexes, we immunoprecipitated p21 and found that cyclin D1, Cdk2, Cdk4, and Cdk6 were all pulled down (Fig. 4*D*). Neither kinase activities nor levels of any of the investigated proteins were altered in control KMT cells cultured in the absence or presence of Zn^{2+} (data nor shown). Finally, we examined changes in Rb phosphorylation status associated with p21 induction. Treatment with Zn^{2+} caused a shift to faster migrating, underphosphorylated forms of Rb (Fig. 4*E*). Underphosphorylated Rb was detected as early as 3 h after Zn^{2+} addition, peaked at 1 day, and disappeared after 2 days, when the majority of Rb protein was again found in its hyperphosphorylated form. This kinetics of Rb hypophosphorylation correlates with the kinetics of p21 induction (Fig. 2*C*).

A parallel analysis was performed with Kp27 cells, where we also found a dramatic reduction of Cdk2 kinase activity following induction of p27 (Fig. 5, *A* and *B*). p27 was present in Cdk2 complexes (Fig. 5*A*), and total Cdk2 protein levels were also somewhat lowered (Fig. 5*C*). Thus, p27 can reduce Cdk2 activity through a direct inhibitory interaction as well as by repressing its expression levels. Expression of p27 also caused a significant inhibition of Cdk4, Cdk6, and cyclin D1-associated kinase activities (Fig. 5*A*). The quantification of the Cdk activities (Fig. 5*B*) confirmed that the Cdk2 and Cdk4 inhibition provoked by p27 was comparable with that of p21 (compare Fig. 4*B* and 5*B*, 24 h) and that, in contrast to p21 (Fig. 4*B*), p27 also potently inhibited Cdk6. p27 induction did not affect overall Cdk4 and Cdk6 protein levels (Fig. 5*C*). Furthermore, immunoprecipitation of p27 pulled down Cdk2, Cdk4, Cdk6, and cyclin D1 proteins (Fig. 5*D*). Induction of p27 elicited the accumulation of Rb in its hypophosphorylated form, and this re-

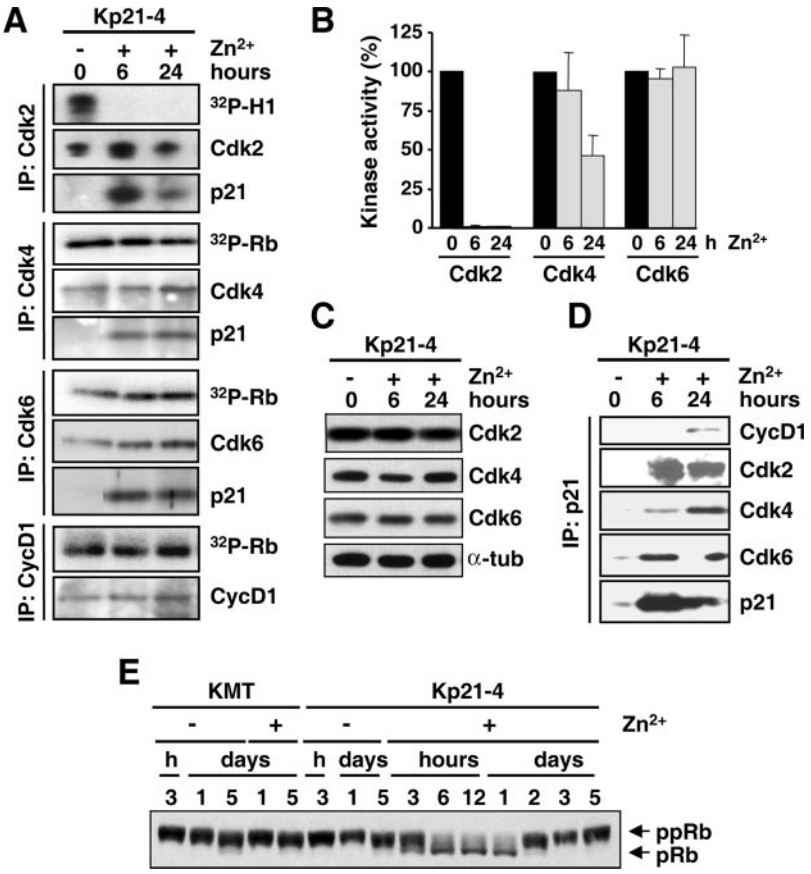

FIG. 4. **Effect of ectopic p21 on activity of G_1 Cdks and Rb phosphorylation status.** *A*, cell extracts were immunoprecipitated (*IP*) with anti-Cdk2, anti-Cdk4, anti-Cdk6, or anti-cyclin D1 antibodies as indicated. Cdk2 activity was assayed using histone H1 as substrate, and Cdk4, Cdk6, and cyclin D1-associated kinase activities were assayed using Rb as substrate. The presence of Cdk2, Cdk4, Cdk6, and cyclin D1 proteins in the immunoprecipitated kinase assays was assessed by subsequent immunoblotting (*middle panels*), as were the levels of p21 present in the immunoprecipitates (*bottom panels*). *B*, quantification of histone H1 or Rb phosphorylation normalized by Cdk immunoprecipitated levels representing the means ± S.E. of three experiments performed as in *A* and plotted as a percentage of starting activity. *C*, Cdk2, Cdk4, and Cdk6 protein levels present in total extracts from Kp21-4 cells prior to and after Zn^{2+} addition, as detected by immunoblotting. *α-tub*, α-tubulin. *D*, association of p21 with Cdk complexes. Whole cell extracts of Kp21-4 cells incubated with or without Zn^{2+} were immunoprecipitated with an anti-p21 antibody and subsequently immunoblotted with antibodies against the indicated proteins. *E*, phosphorylation status of Rb in KMT and Kp21-4 cells grown in the absence or presence of Zn^{2+} was determined by immunoblotting.

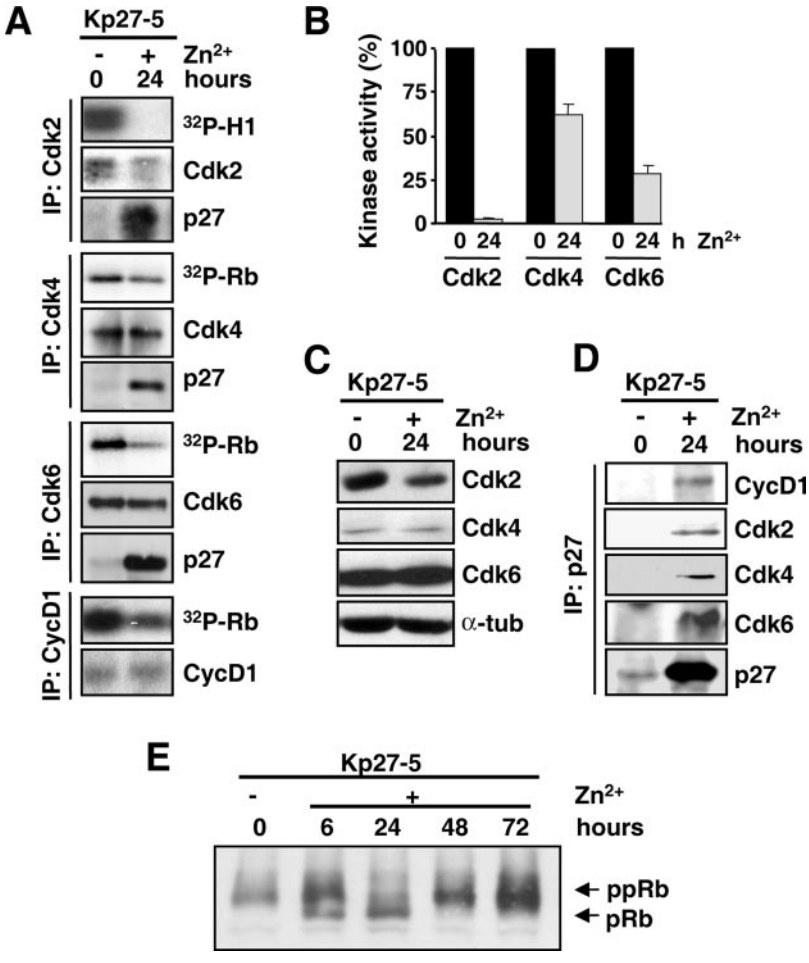

FIG. 5. **Effect of ectopic p27 on activity of G_1 Cdks and Rb phosphorylation status.** Kp27-5 cells were grown in the absence or the presence of Zn^{2+}, and the activities of Cdk2, Cdk4, Cdk6, and cyclin D1-associated kinases (*A* and *B*), levels of G_1 Cdks in total extracts (*C*), association of Cdks with p27 complexes (*D*), and phosphorylation of Rb (*E*) were analyzed as described in the legend for Fig. 4. *α-tub*, α-tubulin.

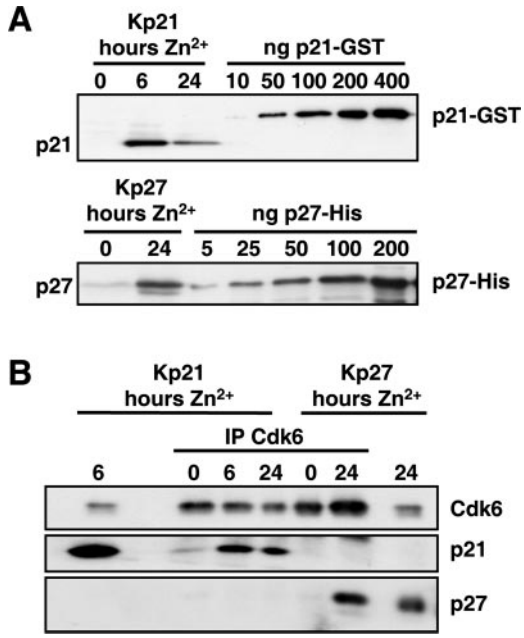

FIG. 6. **Quantification of p21 and p27 levels bound to Cdk6 complexes.** *A*, similar protein amounts from Kp21 and Kp27 cells untreated or treated with Zn^{2+} for the indicated times were analyzed by immunoblotting with anti-p21 and anti-p27 antibodies. For comparison, neighboring lanes were loaded with increasing amounts of recombinant p21 or p27 proteins. *B*, the indicated extracts were immunoprecipitated (*IP*) with anti-Cdk6 antibody, and the amounts of coimmunoprecipitated p21 and p27 were estimated by immunoblotting. Note that the amount of Cdk6 immunoprecipitated from Zn^{2+}-treated Kp27-5 cells at 24 h was about 2.5-fold higher than the level immunoprecipitated in Zn^{2+}-treated Kp21-4 at 6 h.

sponse was closely correlated with the inhibition of Cdk kinase activities (Fig. 5*E*). Taken together, these data suggest that expression of p21 blocks the cell cycle of K562 cells by strongly inhibiting Cdk2 and to a lesser degree Cdk4, without impairing Cdk6 activity. However, p27 blocks the cell cycle by inhibiting not only Cdk2 and Cdk4 but also Cdk6.

One possible explanation for the observed differences in the inhibition of Cdk activities would be that p21 and p27 were induced to different levels. Because p21 and p27 were detected immunologically with antibodies that may well have different affinities for their cognate proteins, we quantitatively calibrated the antibodies against known amounts of recombinant p21 and p27 proteins. When we compared by immunoblotting the levels of p21 and p27 expressed in Kp21 and Kp27 cells induced with Zn^{2+} with known quantities of recombinant proteins, we found that at their peak of expression both CKIs were expressed at similar absolute levels in the two cells lines (Fig. 6*A*). We subsequently considered the possibility that the marked difference in susceptibility of Cdk6 to p21 *versus* p27 inhibition may reside in a differential ability to interact with the two CKIs. However, when we compared the coimmunoprecipitation of p21 and p27 with Cdk6 (normalized to the immunoprecipitated Cdk6), we observed that equivalent amounts of p21 and p27 were bound to Cdk6 (Fig. 6*B*). Thus, Cdk6 appeared less susceptible to inhibition by bound p21 than by equivalent amounts of bound p27.

p21 Induces Megakaryocytic Differentiation whereas p27 Induces Erythroid Differentiation—We next investigated whether the block in cell cycle progression elicited by p21 or p27 was accompanied by differentiation. Treatment of Kp21 cells with Zn^{2+} resulted in morphological changes compatible with myeloid/megakaryocytic differentiation in most cells, as observed in Giemsa-stained preparations (Fig. 7*A*). A significant fraction (25–35%) of cells had highly lobulated nuclei typical of megakaryocytes. No morphological changes were observed in KMT or K562 cells after Zn^{2+} treatment. Because polyploidization is unique to megakaryocytes among hematopoietic cells, we investigated Kp21 cells by flow cytometry, and we found a significant population (~16%) with an 8C DNA content, after 3 days of culture in the presence of Zn^{2+} (Fig. 7*B*). Moreover, a fraction of BrdUrd-positive Kp21 cells was observed between the 4C and 8C peaks, indicating that p21 induced endoreduplication in these cells (Fig. 7*B*). Zn^{2+}-treated Kp21 cells formed clusters and adhered to the culture dish surface (data not shown), suggestive of increased expression of adhesion molecules. Indeed, we found that integrin β_3 (GpIIIa), a known marker of megakaryocytic differentiation (37), was up-regulated (Fig. 7*C*). In addition, two other megakaryocyte-specific markers, β_1-tubulin and c-Mpl (38), were expressed upon induction of p21 (Fig. 7*D*). No significant changes in the expression of these genes were detected in KMT cells (data not shown). Even a short exposure to p21 is sufficient for a commitment to differentiation, because polyploidization was also observed after a brief 6-h pulse of Zn^{2+} (data not shown). In addition, a small fraction of cells (12%) with cytological features of early erythroid differentiation was also observed after longer periods of Zn^{2+} treatment (5 days) in Kp21 cells. These cells showed cytological features of early erythroid differentiation but no terminal differentiation (Fig. 7*A*), and were also observed at a low level (5%) in control K562 and KMT cells (not shown). We conclude that expression of p21 induces predominantly megakaryocytic differentiation in K562 cells.

Induction of p27 did not elicit morphological features of megakaryocytic differentiation or the expression of megakaryocytic markers (data not shown). In contrast, we observed a significant population of cells with cytological features of early erythroid differentiation (proerythroblasts and basophilic erythroblasts). Indeed, Zn^{2+} treatment of Kp27 cells caused a marked accumulation of benzidine-positive cells (50%), indicative of a high hemoglobin content (Fig. 8*A*), which was confirmed by a significant accumulation of ϵ-globin mRNA (Fig. 8*B*). Glycophorin A, a well documented erythroid-specific gene, was induced 9-fold relative to control KMT cells (Fig. 8*C*). In agreement with the previously observed reversible effect of p27 on cell cycle progression, erythroid differentiation was also reversible, because cells not only resumed proliferation but also lost erythroid markers when Zn^{2+} was removed from the media after 24 h (data not shown). We conclude that expression of p27 triggers erythroid differentiation but not megakaryocytic maturation in K562 cells.

The K562 cell line can be differentiated into the erythroid lineage by treatment with cytosine arabinoside (ARA-C) (31) and into the megakaryocytic lineage by staurosporine (30). Most interestingly, we found that in response to these chemicals p21 and p27 proteins were differentially up-regulated at early times; p21 increased in cells committed toward megakaryocytic differentiation, and p27 was up-regulated in cells differentiating along the erythroid lineage (Fig. 9). The differentiation pathways induced in K562 by staurosporine and ARA-C were assessed in parallel by cell morphology and lineage-specific marker expression (data not shown). Comparison of mRNA and protein expression showed that p21 and p27 were regulated mostly by post-transcriptional mechanisms. Thus, the changes on p27 protein levels after differentiation are in contrast with the almost constitutive expression of p27 mRNA, providing an extreme example of the intense post-transcriptional regulation of p27, already described in other models (39). The data also demonstrated that p27 but not p21 protein accumulated in high density growth-arrested K562 cells cultures (Fig. 9, *lanes* C_5). Thus, the expression of p21 and p27 during

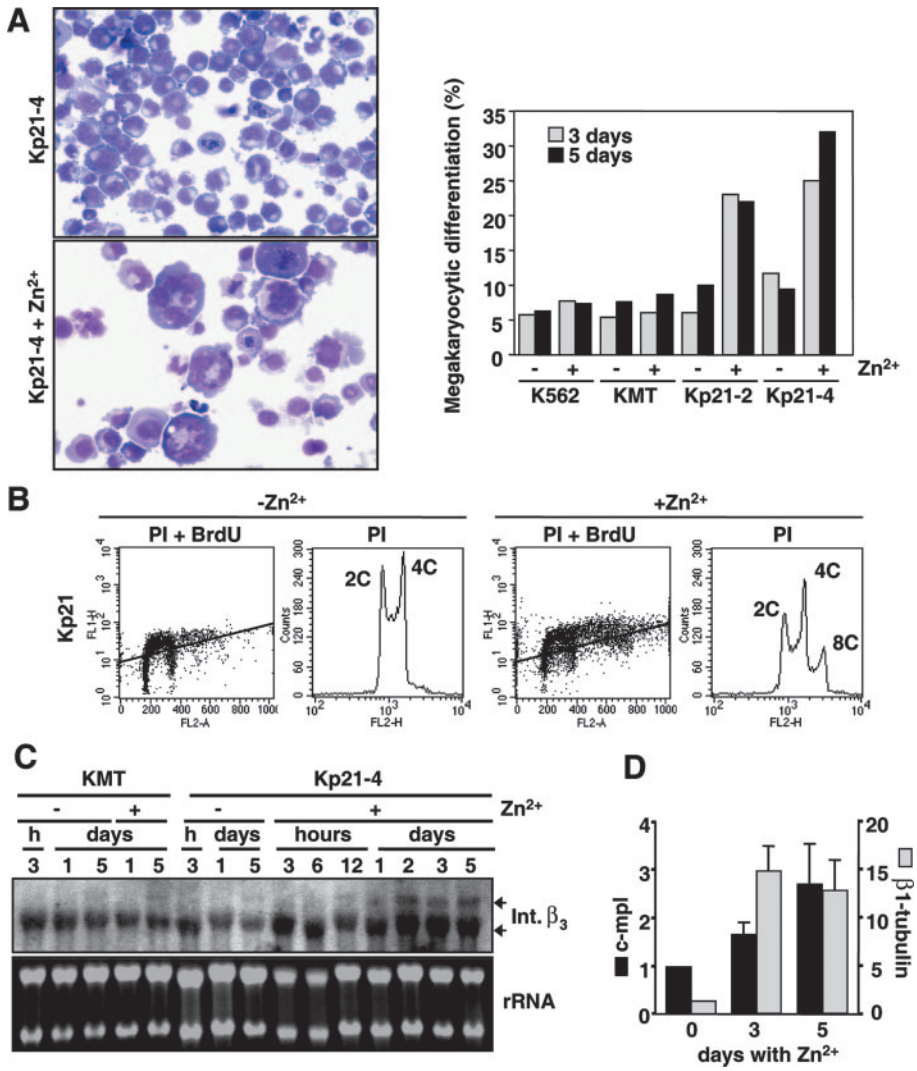

FIG. 7. **Differentiation of K562 cells induced by ectopic p21 expression.** *A*, morphology of Kp21-4 cells after p21 induction. *Left panel*, staining with May-Grünwald-Giemsa of untreated cells (*upper panel*) and cells treated for 5 days with 75 μM $ZnSO_4$, showing large megakaryocyte-like cells (*lower panel*). Both preparations were photographed at the same original amplification (×400). *Right graph*, percentage of megakaryocyte-like cells in the indicated cell lines (n = 3). *B*, two parameter flow cytometric analysis illustrating endoreduplication and polyploidization of Kp21-4 cells in response to Zn^{2+} treatment for 3 days. *Bars*, limit of background fluorescence corresponding to cells cultured in the absence of BrdUrd (*BrdU*) and incubated with antibody. *PI*, propidium iodide. *C*, Northern hybridization analysis of integrin β_3 (*Int.* β_3) in KMT and Kp21-4 cells incubated with or without Zn^{2+}. The positions of the 5.9- and 4.1-kb transcripts of integrin β_3 are indicated. *D*, real time reverse transcription-PCR analysis of c-Mpl and β_1-tubulin mRNA expression in Kp21-4 cells treated with 75 μM $ZnSO_4$. Data refer to relative expression values normalized to the levels of ribosomal protein S14 in each case. Values shown are means ± S.E. (n = 3).

the commitment response of K562 cells is in complete agreement with the differentiation programs induced by the ectopic up-regulation of the individual CKIs.

p21 Induces Weak Accumulation of p27 Protein That Can Mediate Limited Erythroid Differentiation—Our results clearly indicate that p21 is able to trigger a megakaryocytic differentiation program; however, its involvement in erythroid differentiation is less clear because p21 induction also resulted in a small fraction of cells with erythroid characteristics. Because it has been reported that ectopically expressed p21 can up-regulate p27 (40), we considered whether a p21-mediated up-regulation of p27 could in turn lead to a low level of erythroid differentiation. Indeed, we found that a moderate elevation of p27 protein levels occurred in Zn^{2+}-treated Kp21-4 cells (Fig. 10*A*). Similar results were also observed in other Kp21 clones (data not shown). In contrast, the induction of p27 in Kp27 cells did not change the expression of p21.

To assess directly the functional significance of p27 up-regulation in Kp21 cells, we tested the effects of depletion of p27 by antisense mRNA. Kp21-4 cells stably expressing a transfected p27 antisense construct (designated Kp21-p27AS) responded to Zn^{2+} treatment by up-regulating p21 to the same extent as parental cells (Kp21-4), but the up-regulation of p27 was effectively attenuated (Fig. 10*B*). Remarkably, the reduced expression of p27 protein was correlated with a significant decrease of cells committing to erythroid differentiation, as evaluated with the erythroid markers of ϵ-globin (Fig. 10*C*) and glycophorin A (Fig. 10*D*). However, the extent of megakaryocytic marker expression, such as polyploidization or induction of c-Mpl expression, was not changed by p27 depletion (not shown). These data show that p27 is responsible for the low level of erythroid differentiation elicited by induction of p21. We conclude that p21 and p27 play distinct roles in differentiation; p21 regulates the megakaryocytic pathway, and p27 acts exclusively in the erythroid lineage.

DISCUSSION

p21 and p27 have been reported to play redundant roles in differentiation of several myeloid cell lines (14, 15). However, these studies employed monopotent cell lines where the differentiation was constrained along a single lineage, thus preventing additional functions of p21 or p27 from being observed. We use here a multipotent cell differentiation system (K562 cells) in which we have engineered temporal control of ectopic p21 and p27 expression in the absence of any additional differentiation agents. Most importantly, the expression levels of p21 and p27 achieved upon induction were similar. Our results in this model system clearly demonstrate distinct roles of these CKIs; p21 promotes megakaryocytic differentiation, and p27 induces erythroid maturation of K562. These differential effects are summarized in Fig. 11.

We have found that p21 triggers megakaryocytic maturation in the majority of K562 cells, in agreement with results using other cell myeloid lines (20, 21). Most interestingly, p21 protein

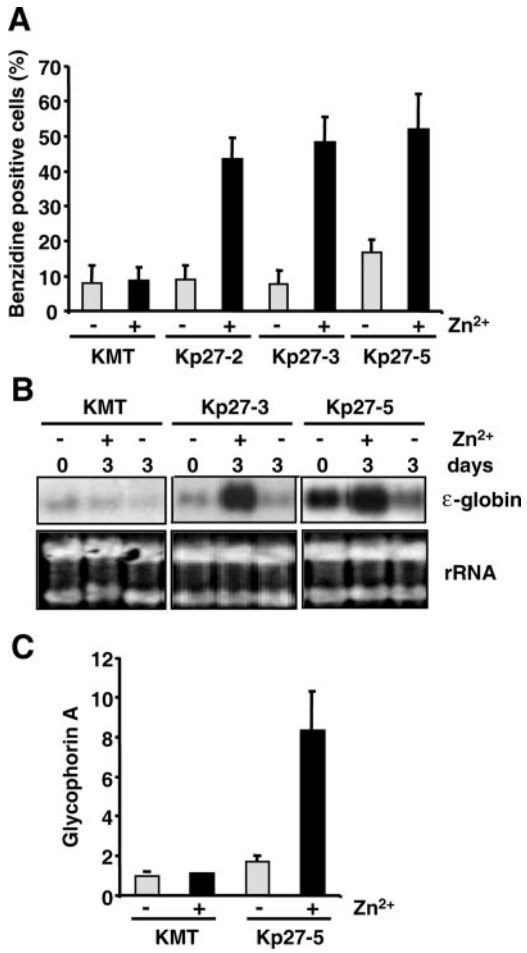

FIG. 8. **Differentiation of K562 cells induced by ectopic p27 expression.** *A*, benzidine staining of KMT and Kp27-2, -3, and -5 cell lines cultured with or without Zn^{2+} for 4 days. Values shown are means ± S.E. ($n = 3$). *B*, Northern hybridization analysis of ϵ-globin mRNA in KMT, Kp27-3, and Kp27-5 cells incubated with or without Zn^{2+}. *C*, real time reverse transcription-PCR analysis of glycophorin A mRNA expression in KMT and Kp27-5 cells induced for 3 days with or without Zn^{2+}. Data refer to relative expression values normalized to the levels of ribosomal protein S14 in each case. Values shown are means ± S.E. ($n = 3$).

expression was only transiently induced in our experiments, which is consistent with reports of p21 down-regulation just prior to terminal differentiation in myeloid cells (29), suggesting that down-regulation of p21 may be necessary for terminal differentiation (15). Transient induction of p21 was also observed during the differentiation of $CD34^+$ cells along a megakaryocytic lineage (28), as well as in other differentiation models (14). This raises the possibility that after the initial induction, the down-regulation of p21 may be required to permit progression to a terminally differentiated state. Whether sustained expression of p21 can block the expression of megakaryocyte-specific markers is currently under investigation.

Previous studies have shown that p27 plays a role in the growth arrest that accompanies erythroid differentiation of mouse erythroleukemia cells, but in contrast to our results, no differentiation was reported after p27 overexpression (41, 42). However, in that model the induction of p27 did not result in the inhibition of Cdk6 activity (42), and as discussed below, the differential regulation of Cdk6 could be responsible for p27-mediated differentiation in K562. It should be noted that a previous report (43) did not find a link between p27 induction and erythroid differentiation in K562 cells. Although the reason for this difference is not clear, it may be explained by the shorter time frame of the differentiation experiments (48 h) used in that report or by the higher p27 expression levels achieved in our studies. Consistent with the latter suggestion, we observed a clear dose-response relationship, such that lower doses of Zn^{2+} (leading to lower levels of p27 expression) resulted in reduced erythroid differentiation. Moreover, the small increase in erythroid markers observed in p21-expressing cells appears to be caused by a coupled up-regulation of p27, because this effect was abrogated by expression of a p27-antisense construct.

Thus the K562-based model system established here supports the hypothesis that p21 and p27 possess unique and not interchangeable biological activities in differentiation. Most importantly, the distinct phenotypes induced by ectopic p21 and p27 are also completely consistent with their expression patterns during chemically induced differentiation of K562 toward megakaryocytic and erythroid differentiation in response to staurosporine and ARA-C, respectively. Consistently, p27 but not p21 is expressed in erythroid progenitors (44), and p21 but not p27 is highly expressed in cycling megakaryocytes (28).

A further interesting difference between p21 and p27 in K562 cells is their effects on cell cycle progression. We found that both p21 and p27 blocked G_1 progression in K562. This G_1 arrest is likely the result of the severe inhibition of Cdk2 activity and the concomitant loss of Rb phosphorylation. However, p27 was more efficient than p21 in eliciting the G_1 arrest, and the strong p27-mediated inhibition of Cdk6 activity may contribute to this effect. In contrast to p27, expression of p21 also caused strong G_2 arrest. The involvement of p21 in the regulation of the G_2/M transition has been noted in previous reports (45–47). Most interestingly, after 2 days of Zn^{2+} treatment of Kp21 cells, p21 expression reverted to low levels, and Rb was again hyperphosphorylated, at which point a significant population of the cells underwent DNA replication without an intervening mitosis. Because endoreduplication was not observed in p27-expressing cells when p27 was down-regulated by removal of Zn^{2+}, it is unlikely that inactivation of Rb *per se* is responsible for the endoreduplication. It appears that cells arrested in G_2 by p21 expression lose the ability to resume a normal cell cycle progression. Indeed, several proteins involved in mitotic checkpoint control and the reinitiation of DNA synthesis in the same cell cycle have been shown to be inhibited by p21 (48, 49).

Our findings also demonstrate a novel and striking difference in the ability of p21 and p27 to regulate the *in vivo* activity of Cdk6 complexes (Fig. 11). We observed that p27 binds and inhibits Cdk6 activity, whereas essentially identical amounts of p21 bound to Cdk6 failed to cause significant inhibition. A similar difference has been reported for Cdk4 complexes using recombinant proteins, although in that case p21 was the more potent inhibitor (4). The fact that K562 are deficient for $p16^{INK4a}$ and $p15^{INK4b}$ (32) rules out a possible involvement of these proteins in the differential inhibition of Cdk4 and Cdk6 by p21 and p27 in this model.

Are the different effects of p21 and p27 on Cdk6 activity responsible for the distinct differentiation pathways induced by these CKIs? Most interestingly, Cdk6 has been implicated as being responsible for the differentiation block and uncontrolled proliferation of leukemic erythroblasts (50). Also, ectopic expression of $p16^{INK4a}$ in K562 results in erythroid differentiation (51), and down-regulation of Cdk6, but not of Cdk4, accompanies erythroid differentiation of MEL cells (42). Moreover, it has been reported recently that Cdk6-deficient mice show improper hematopoietic homeostasis and reduced number of red blood cells (52). The repression of Cdk6 in K562 cells may thus sensitize them toward erythroid differentiation

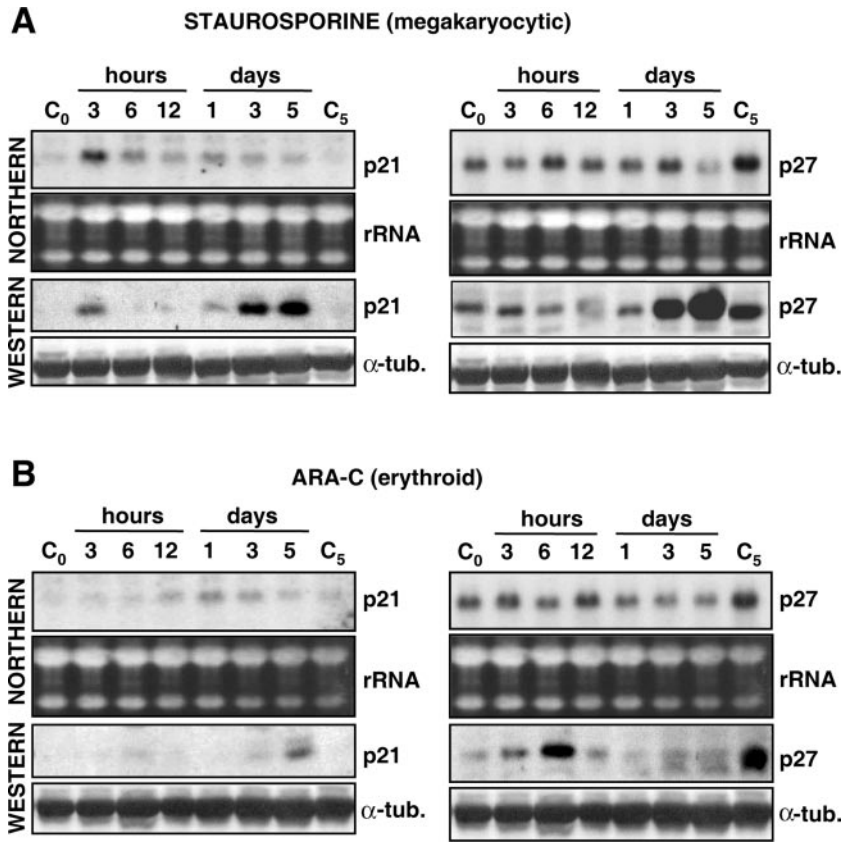

FIG. 9. **Expression of p21 and p27 during chemically induced differentiation of K562 cells.** *A*, K562 cells were exposed to 100 nM staurosporine to induce megakaryocytic cell differentiation. At the indicated times, the expression of p21 and p27 mRNA was analyzed by Northern hybridization (*upper panels*). *Lower panels* show the expression of p21 and p27 proteins at various times after staurosporine addition as determined by immunoblotting. *B*, K562 cells were exposed to 1 μM ARA-C to induce erythroid cell differentiation. At the indicated times, the expression of p21 and p27 mRNA (*upper panels*) and protein (*lower panels*) was analyzed as described above. C_0 lane represents control cells prior to drug addition, and C_5 *lane* corresponds to cells maintained in culture for 5 days. *α-tub*, α-tubulin.

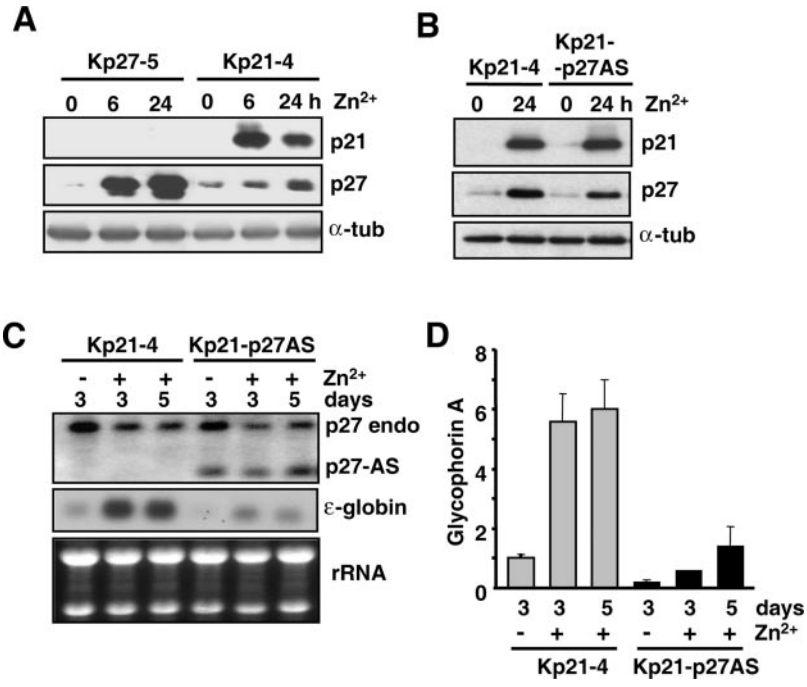

FIG. 10. **Attenuation of p21-induced erythroid differentiation by p27 antisense.** *A*, immunoblot showing p21 and p27 protein expression in Kp21-4 and Kp27-5 cells. Cells were cultured in the presence (+) or the absence (−) of Zn^{2+} for the indicated times. The filters were blotted with antibodies directed against p21, p27, and α-tubulin (*α-tub*). *B*, p21 and p27 expression in Kp21-4 and Kp21-p27AS cells (Kp21 cells expressing a p27 antisense construct). By film densitometry there is a 37% reduction in p27 expression in the Kp21-p27AS cells. *C*, Northern hybridization analysis of Kp21-4 and Kp21-p27AS cells showing expression of endogenous p27 and p27 antisense transcripts (*p27-AS*). The expression of the erythroid-specific gene ε-globin is also shown. *D*, real time reverse transcription-PCR analysis of glycophorin A mRNA in Kp21-4 and Kp21-27AS cells with or without Zn^{2+} as indicated. Data refer to relative expression values normalized to the levels of ribosomal protein S14 in each case. Values shown are means ± S.E. (n = 3).

in response to inhibition by p27. However, both p21 and p27 inhibit Rb phosphorylation, leaving open the hypothesis that the distinct differentiation lineages induced by p21 and p27 depend on activities unrelated to cell cycle control, including gene expression regulation, the importance of which is increasingly recognized (12, 13, 53).

Because p21 and p27 are highly similar in their Cdk inhibitory domains, the basis for the differential sensitivity of Cdk6 is not clear. One possibility may be that other proteins may interact with p21-Cdk6 complexes but not with p27-Cdk6 complexes and antagonize the inhibition by one but not the other CKI. One example of such a mechanism is the SET protein, which regulates the inhibitory effect of p21 on cyclin E/Cdk2 activity (54). Along the same lines it is possible that specific post-translational modifications may protect the Cdk-cyclin core binary enzyme from CKI-mediated inhibition. Thus, as the interacting proteins and post-transcriptional modifying systems may vary with the cell type, it is likely that the differential effects of p21 and p27 on Cdk activity are cell type-dependent. Finally, despite high sequence similarity, important

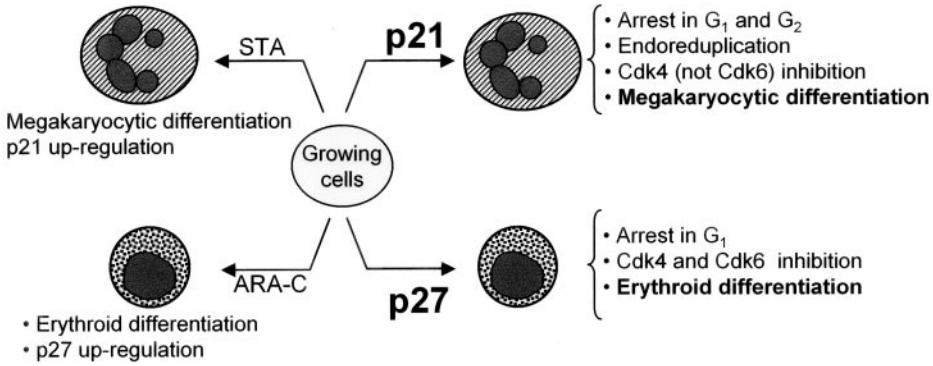

FIG. 11. **Summary of the effects of p21 and p27.** The major effects of the transient induction of p21 and p27 on cell cycle and on differentiation in K562 cells are indicated in each case. The indicated effects are discussed in detail in the text. The differentiated phenotype induced by p21 and p27 is consistent with the expression of each protein during megakaryocytic or erythroid differentiation induced by staurosporine (*STA*) and ARA-C, respectively.

differences in the specific interactions between individual CKIs and Cdk-cyclin complexes may well exist. For example, p21 may contact only the cyclin subunit in the Cdk6 complex, whereas p27 may interact with both the cyclin and the Cdk domains, thus more significantly impairing kinase activity. In the absence of structural information, the mechanisms by which p21 and p27 exert distinct regulatory effects on Cdk6 complexes and whether this effect is responsible for the striking difference in the induced differentiation lineage await further study.

Acknowledgments—We are grateful to James DeCaprio, Katya Ravid, Barrett Rollins, Ramesh Shivdasani, and Piotr Sicinski for helpful discussions; Angel Corbi, Silvio Gutkind, Manuel Serrano, and Frank Rausher for plasmids; and Nicholas Cowan for anti-tubulin antibody. We thank Rosa Blanco, Pilar Frade, and María Aramburu for technical assistance.

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