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Regulation of CDC6, Geminin, and CDT1 in Human Cells that Undergo Polyploidization

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Endomitosis is the process by which mammalian megakaryocytes become polyploid during terminal differentiation. As in other endoreplicating cells, cyclin-cdk complexes are distinctly regulated, probably to overcome the strict mechanisms that prevent rereplication in most somatic cells. We have asked whether key factors involved in the assembly and licensing of replication origins are equally regulated during endomitosis. Cdc6, cdt1, and geminin expression was analyzed during differentiation of two human megakaryoblastic cell lines, HEL and K562, which respectively do and do not establish endoreplication cycles. Geminin was downregulated, whereas cdt1 levels were maintained upon differentiation of both cell lines, independently of whether cells entered extra S-phases. In contrast, cdc6 was present and remained nuclear only in differentiated endoreplicating cells. Interestingly, cdc6 protein expression was reestablished in K562 cells that underwent endomitosis after transient or stable cyclin E overexpression. The high levels of cyclin E reached in these cells appeared to influence the stabilization of cdc6 protein rather than its RNA transcription rate. Finally, cdc6 overexpression drove HEL cells into endoreplication cycles in the absence of differentiation stimuli. Our results show that both cdt1 and cdc6 are differentially regulated during megakaryocytic differentiation and suggest an active role of cdc6 in endomitosis.

INTRODUCTION

In eukaryotic cells, DNA replication must be restricted to once per cell cycle. However, some cell types, from plants to mammals, physiologically become polyploid by establishing rereplication cycles as part of their differentiation programs. One of the few adult cell types with this peculiar feature is the mammalian megakaryocyte. During final maturation stages, megakaryocytes repeatedly replicate their nuclear DNA and undergo an abortive mitosis that lacks karyo- and cytokinesis, in a process traditionally known as endomitosis (Nagata *et al.*, 1997; Vitrat *et al.*, 1998). It is conceivable that in these cells, at least some of the processes involved in the prevention of replication reinitiation must be altered.

The mechanisms that guarantee the strict alternation of S and M phases within a mitotic cycle are remarkably conserved from yeast to mammals. They comprise a finely tuned interplay between cyclin-dependent kinases, responsible for the proper progression through G1, S, G2, and M phases, and protein complexes involved in building up and licensing the origins of replication (for review, see Diffley and Labib, 2002). During G1 phase, prereplication complexes (pre-RC) are assembled, then origin firing is triggered at the entrance into S phase, and once replication has been initiated, origins remain silenced until daughter cells progress onto the next G1 phase. At the end of mitosis, assembly of prereplication complexes starts by separately recruiting two factors, cdc6 and cdt1, to the origin recognition complex (ORC; Cocker *et al.*, 1996; Coleman *et al.*, 1996; Blow and Tada, 2000; Maiorano *et al.*, 2000; Nishitani *et al.*, 2000). ORC is formed by six distinct subunits, and their binding to DNA determine, at least in part, the identity of the replication origin (Quintana and Dutta, 1999). The binding of cdc6 and cdt1 is essential to form the pre-RC complex, because they both promote the loading of the license complex of mini-chromosome maintenance proteins, mcm 2–7 (Lei and Tye, 2001). MCM complexes are required both for initiation and elongation phases, probably because of their unwinding helicase activity at replication forks (Ishimi, 1997; Patel and Picha, 2000). The pre-RC is converted into a preinitiation complex (pre-IC) by further recruiting of additional factors such as cdc45 and Dbf4-cdc7 complex (Leatherwood, 1998; Lei and Tye, 2001). Cdc7 kinase could be responsible, together with cyclin E-cdk2, for triggering DNA replication (Roberts *et al.*, 1999; Sclafani, 2000). Cdc6 and

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cdt1 are only necessary for initiation, and it is believed that they are released from the pre-IC at the onset of S phase (Diffley and Labib, 2002). In metazoans, cdc6 is stable during S phase. However, it is exported from the nucleus, probably after being phosphorylated by cyclinA-cdk2 (Saha *et al.*, 1998; Findeisen *et al.*, 1999; Jiang *et al.*, 1999; Petersen *et al.*, 1999). Thus, cdc6 activity seems to be controlled through G1/S cyclins-cdk complexes. In contrast, cdt1 activity appears to be regulated through interaction with geminin (Wohlschlegel *et al.*, 2000; Tada *et al.*, 2001), a potent replication inhibitor the expression of which is regulated in a cell cycle–dependent manner (McGarry and Kirschner, 1998; Bastians *et al.*, 1999). Cdt1 expression seems to remain constant throughout the cell cycle (Nishitani *et al.*, 2001), whereas geminin is proteolysed via the APC/C complex after onset of mitosis (McGarry and Kirschner, 1998). In this way, cdt1-induced MCM binding can only take place in the absence of geminin, i.e., from late mitosis to early S phase, when geminin begins to be detected in proliferating cells (McGarry and Kirschner, 1998; Nishitani *et al.*, 2001).

Most biochemical studies aimed at understanding the molecular mechanisms by which megakaryocytes are able to overcome rereplication restrictions have focused on whether cyclin-cdk complexes are differentially regulated in endomitotic cells. In vitro and in vivo experimental approaches have shown that both G1/S and G2/M cell cycle transitions have to be regulated for megakaryocytes to achieve polyploidization (for a recent review see Ravid *et al.*, 2002). For instance, both cyclins A and B are expressed and temporally regulated as in a mitotic cycle (Datta *et al.*, 1996; Garcia and Cales, 1996; Vitrat *et al.*, 1998; Garcia *et al.*, 2000; Bornstein *et al.*, 2001). In contrast, both cyclinD3-cdk2, and cyclinE-cdk2 complexes have been proposed as the most likely candidates to drive megakaryocytic polyploidization (Wang *et al.*, 1995; Garcia and Cales, 1996; Matsumura *et al.*, 2000). Cyclin D3 expression is markedly higher in megakaryocytes (Zimmet *et al.*, 1997), and cyclin E appears to be differentially regulated in megakaryocytic cells undergoing endoreplication cycles (Garcia and Cales, 1996; Datta *et al.*, 1998; Bornstein *et al.*, 2001). In fact, cyclin E expression is maintained in cells that have accomplished the endoreplication S phase, and when overexpressed, this cyclin is able to drive nonendoreplicating megakaryoblastic cells into endomitosis (Garcia *et al.*, 2000). It seems, therefore, that basal cell cycle machinery is differentially controlled during megakaryocytic endoreplication. However, the mechanisms by which such cyclin-cdk alterations affect the replication initiation machinery are unknown.

Our work was aimed at determining whether key factors involved in both replication firing and refiring prevention are differentially regulated during megakaryocytic endoreplication. Data are presented that show how cdc6, cdt1, and geminin are regulated in two megakaryoblastic cell lines, HEL and K562, which have similar responses to differentiation stimulus in terms of megakaryocytic differentiation, but only one of which, the HEL cell line, acquires a polyploid phenotype.

MATERIALS AND METHODS

Cell Culture

HEL, K562, and derived HA1 and KEB cells were cultured in RPMI 1640 medium (Life Technologies, Paisley, UK) supplemented with 10% (vol/vol) fetal calf serum (BioWhitaker, Verviers, Belgium), 2 mM L-glutamine (Life Technologies) and 60 mg/ml gentamicin (Normon Laboratories, Madrid, Spain). G418 antibiotic (Life Technologies) was added to HA1 and KEB cells culture medium to a final concentration of 200 μg/ml. HeLa cells were cultured in DMEM (Life Technologies) medium supplemented with 10% (vol/vol) fetal calf serum (Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin (both from Life Technologies). Cells were maintained at 37°C under 5% CO_2/95% air in a humidified incubator. To induce megakaryocytic differentiation, $0.15–0.20 \times 10^6$ cells per ml were grown in the presence or absence of 10^{-8} M *o*-tetradecanoylphorbol 13-acetate (TPA; Sigma, Madrid, Spain) for the indicated times. For S-phase synchronization, HeLa cells were cultured in the presence of 2.5 mM thymidine (Sigma, St. Louis, MO) for 25 h, washed once with phosphate-buffered saline (PBS), and then allowed to grow for an additional 2 h in fresh medium. For metaphase synchronization, HeLa cells were cultured in presence of nocodazol (Sigma) at 50 μg/ml for 14 h.

Constructs and Oligonucleotides

For pMTcdc6, a 1.8-kb fragment encoding full-length human cdc6 cDNA was isolated by partial digestion with *Xho*I and *Xba*I from pBSK-cdc6 and subcloned into pCS2MT (Rupp *et al.*, 1994) multicloning site in order to construct an myc-tagged form of the protein. A 2-kb *Bam*HI fragment was then subcloned into pLZR-CMV-IRES-ΔNGFR (Abad *et al.*, 2002) in order to construct pLZR-cdc6. A 1.8-kb *Bam*HI/*Not*I fragment from pBS-GSTcdc6 5XA (Herbig *et al.*, 2000) encoding a phophorylation mutant of human cdc6 was subcloned into pCDNA3.1 (Invitrogen, San Diego, CA), and an *Hin*dIII/*Xba*I partially digested fragment from this plasmid subsequently was inserted into pS72 vector (Promega, Madison, WI). A *Xho*I/*Xba*I fragment was then inserted in the corresponding sites of pCS2MT to obtain pMTcdc6–5xA.

For full-length human geminin cDNA cloning, the reverse transcriptase polymerase (Promega) reaction was performed using total RNA extracted from hydroxyurea-treated HeLa cells. cDNA was amplified by PCR using Pfu-polymerase (Promega). 5′ATGAATCCCAGTATGAAGCAG-3′ and 5′-CTTCGGCAGTAAAATTCTCAA-3′ were used as upper and lower primers, respectively. The resulting 0.7-kb amplimer was purified and subcloned into the *Eco*RV site of pZEro-2.1 (Invitrogen). PGEX-cdt1 encoding full-length human cdt1 cDNA was obtained from Dr. Dutta. pCDNA3-cyclinE plasmid construction has been described elsewhere (Garcia *et al.*, 2000)

Northern Blot

Total RNA was extracted using TRIzol Reagent (Life Technologies), following the procedure described by the manufacturer. RNA samples were denatured, electrophoresed in 1.1% agarose-formaldehyde gels containing 0.1 mg/ml ethidium bromide and blotted onto nylon membranes (Hybond-N; Amersham, Barcelona, Spain). Ethidium bromide (Sigma) staining of 28S and 18S rRNAs was routinely checked before and after blotting as a control of sample loading and RNA transfer, respectively. RNA blots were prehybridyzed, then hybridyzed with excess (^{32}P)-labeled probes, washed under highly stringent conditions, and auto-radiographed. The following probes were used: 1.9-kb human cdc6 *Bam*HI-*Sal*I fragment from pBSKcdc6 plasmid; 1.9-kb human cdt1 *Not*I-*Eco*RI fragment from pGEX-cdt1; 0.7-kb human geminin *Not*I-*Pst*I fragment from pZErO-geminin, and the entire pTRI-RNA28S plasmid, which hybridyzes to 28 S rRNA (Ambion Inc., Austin, TX). The fragments were labeled to $\sim 10^9$ cpm per μg of DNA with [α-^{32}P]dCTP (3000 Ci/mmol; New England Nuclear, Boston, MA) using the Prime-It II random priming labeling kit (Stratagene, La Jolla, CA).

Transient Transfection and NGFR-positive Cell Immunoselection

Five to 20 million cells were washed and resuspended in serum-free RPMI medium before electroporation at 250 V and 975 μFd with 2 μg of plasmid DNA per one million cells. After 10 min on ice, cells were seeded in serum-supplemented medium. Cells were allowed to recover for 12 h and then divided into two aliquots, one of which was treated with TPA.

For pLZR-ΔNGFR transfection experiments, cells were harvested 48 h after TPA treatment and washed in PBS/1%BSA. Before immunoselection, an aliquot from either control or cdc6-transfected cells was incubated with anti-human NGFR (PharMingen, San Diego, CA) and afterward with an anti-mouse FITC antibody (PharMingen) to determine by flow cytometry the extent of ΔNGFR expression (typically 5–10% of cells). For immunoselection, magnetic beads conjugated to anti-mouse IgG through a DNA bridge (Dynal, Oslo, Norway) were pretreated with anti-human NGFR for 15 min at 4°C. Positive cells were then collected by magnetic selection with the anti-NGFR–coupled beads, washed, and released by incubation with a DNAse solution, according to the manufacturer's instructions. Aliquots from whole cell population before immunoselection, cells not bound to αNGFR-beads, and DNAse-released cells (namely, "whole population," "negative cells," and "positive cells," respectively) were collected, and DNA content was assessed by flow cytometry.

Flow Cytometry Analysis of DNA Content

DNA content was determined by staining with 50 μg/ml propidium iodide (Sigma) as previously described (Garcia and Cales, 1996). Cell cycle analysis was performed with a FACScan analyzer and CellQuest software (Becton Dickinson).

Protein Extracts

Total cellular proteins were extracted in Lysis Buffer (20 mM Tris-HCl, pH 7.4; 10 mM EDTA; 100 mM NaCl; 1% Triton X-100) containing protease and phosphatase inhibitors. For phosphatase treatment, 100 μg of total protein was incubated with 25 U of calf intestine alkaline phosphatase (CIAP; MBI Fermentas GmbH, St. Leon-Rot, Germany) for 15 min at 30°C before being subjected to SDS-PAGE. The reaction was stopped by adding Laemmli sample buffer. For nuclear and cytoplasmic fractionation, cells were collected, washed once with PBS and once again with hypotonic buffer (20 mM HEPES, 5 mM potassium acetate, 500 nM $MgCl_2$, and 500 nM DTT). After incubation in the hypotonic buffer for 10 min at 4°C, cells were centrifuged for 5 min at 1800 × *g* and resuspended in a small volume of hypotonic buffer supplemented with protease and phosphatase inhibitors. Cells were homogenized with a Dounce homogenizer (loose pestle) and centrifuged for 5 min at 1800 × *g* at 4°C in order to recover intact cell nuclei. Supernatant containing cytoplasmic proteins was collected and centrifuged for 20 min at 16,000 × *g* at 4°C. The nuclear pellet was washed once in hypotonic buffer and then lysed in hypertonic buffer (20 mM HEPES, 5 mM potassium acetate, 500 nM $MgCl_2$, 500 nM DTT, and 400 mM NaCl) for 1 h and 30 min at 4°C. The lysate was centrifuged and the supernatant was collected. Total, nuclear, and cytoplasmic fractions were snap-frozen and stored at −70°C until further analysis.

Western Blot Analysis

Protein extracts were subjected to SDS-PAGE and proteins transferred to BioTrace PVDF membranes (Pall Corporation, Ann Arbor, MI) for 1 h at 2 mA/cm^2 on a semidry transfer apparatus (Amersham). Ponceau staining was routinely performed on membranes to check sample loading control. After blocking in PBS containing 0.1% Tween 20 (T-PBS) and 5% skimmed dry milk, filters were incubated overnight at 4°C with the appropriate primary antibody diluted in T-PBS. Antibodies used were as follows: anti-human cdc6 mouse mAb (Ab3; Oncogene, Darmstadt, Germany) at a 1:500 dilution; anti-human cdt1 rabbit polyclonal antibody (Nishitani *et al.*, 2000) at a 1:2500 dilution; anti-human geminin goat polyclonal antibody (C-16; Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1000 dilution; anti-human cyclin E mouse mAb (PharMingen) at a 1:1000 dilution; and anti-human cyclin A rabbit polyclonal antibody (Santa Cruz Biotechnology) at a 1:1000 dilution. Anti-IκBα (C-21; Santa Cruz Biotechnology) and anti-PCNA (Signet, Redham, MA) were used as nuclear and cytoplasmic fractionation controls at 1:1000 and 1:2000 dilutions, respectively. Anti-heat shock transcription factor-1 (StressGen Biotechnologies Corp., Victoria, British Columbia, Canada) was used at 1:2000 dilution as a positive control for CIAP treatment. After washing and incubation with an appropriate secondary antibody conjugated to horseradish peroxidase (Dako, Glostrup, Denmark), signals were detected using the enhanced chemiluminescence system (Pierce, Rockford, IL).

Immunofluorescence Microscopy Analysis

After transfection with pCSMTcdc6 or pCSMT, KEB cells were treated with $10^{[minoa]8}$M TPA for 48 h. Differentiated cells were then collected, washed once with PBS and cytospun on to glass slides. Cells were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature, permeabilized with 1% T-X100, and washed twice in PBS. After quenching in 10 mM glycine for 10 min, cells were incubated in blocking solution (2% BSA; 0,05% Tween 20 in PBS) for 1 h at RT and incubated with anti-human myc 9E10 epitope mAb overnight at 4°C. After washing three times with 0.05% Tween 20 in PBS, cells were incubated with a 1:200 dilution of anti-mouse Ig conjugated to Alexa Fluor 488 (Molecular Probes Europe BV, Leiden, The Netherlands) in blocking solution for 1 h at room temperature. After extensive washing with 0.05% Tween 20 in PBS, nuclei were stained with 1 mg/ml DAPI for 5 min. After final washing, preparations were treated with antifading Vectashield (VectorLabs, Burlingame, CA) and examined under a confocal microscope (TCS-SPII; Leica Microsystems Wetzlar GmbH, Wetzlar, Germany).

RESULTS

Cdc6 Expression Is Differentially Maintained in Endoreplicating Cells

We first examined the steady state levels of cdc6, cdt1, and geminin RNA in differentiated HEL and K562 cells. A Northern blot analysis was carried out with total RNA from exponentially growing or TPA-treated cells, using radiolabeled human cdc6, cdt1, or geminin full-length cDNAs as a probe. As seen in Figure 1A, the detected bands corresponding to cdc6 transcripts (of ~3.3 and 2.5 kb) reached slightly higher levels in differentiated than in exponentially growing HEL cells, whereas they significantly decreased in TPA-treated, nonendoreplicating K562 cells when compared with growing cells. On the other hand, the levels of cdt1 transcripts (of ~3 and 4 kb in size) decreased to similar levels in both endoreplicating and nonendoreplicating cells (Figure 1B), and geminin transcript was profoundly downregulated in both cell lines upon TPA treatment (Figure 1C). Thus, it appears that only cdc6 expression was differentially regulated in HEL versus K562 cells, because cdt1 and geminin transcripts were downregulated upon differentiation of both cell lines.

We wanted to confirm that the differential expression pattern between HEL and K562 cells was also maintained at the protein level. Western blot analysis was then performed by incubating total protein extracts obtained from control and TPA-treated HEL and K562 cells. It could be observed

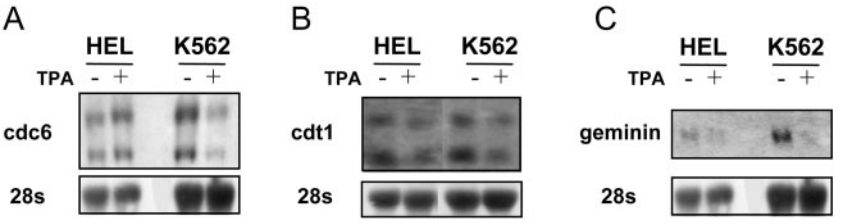

Figure 1. Cdc6 mRNA levels are upregulated in endoreplicating megakaryoblastic cells. HEL and K562 cells were cultured in the absence or presence of 10^{-8} M TPA, and total RNA was extracted. RNA, 20 μg, from exponentially growing cells (−) or cells treated with TPA for 48 h (+) was analyzed by Northern blotting with full-length cdc6 (A), cdt1 (B) and geminin (C) cDNAs as probes. The same blots incubated with a probe recognizing 28S ribosomic RNA are shown as a loading control (note that the blot analyzed with geminin probe was the same used for cdc6 mRNA detection, after washing and rehybridization).

that whereas cdc6 was still present in differentiated HEL cells (Figure 2A, lane 2), the protein could not be detected in nonendoreplicating K562 extracts, at least in the same experimental conditions (Figure 2A, lane 6). To ascertain whether the detected maintenance of cdc6 in HEL cells was related to the establishment of endoreplication cycles, we carried out similar experiments in cell lines derived from HEL and K562 that have lost and gained, respectively, the ability to undergo endomitosis (Figure 2B). In the case of HA1 cells, these ectopically expressing Dm*escargot* HEL cells do not establish endoreplication cycles in response to TPA (Ballester *et al.*, 2001; Figure 2B). Inversely, KEB have been derived form K562 cells by constitutive expression of cyclin E and are able to become polyploid when treated with the differentiating agent (Garcia *et al.*, 2000; Figure 2B). In KEB cells, TPA treatment determines the stabilization of the exogenous cyclin E (Garcia *et al.*, 2000; and as indicated in the corresponding blot, Figure 2A). The Western blot analysis of total protein extracts from growing and differentiated HA1 and KEB cells revealed that cdc6 was present in endoreplicating K562-derived KEB cells, whereas no protein could be detected in TPA-treated HA1 cells extracts. The blots were then reincubated with anti-cdt1 and antigeminin antibodies. According to the TPA-driven downregulation of the corresponding transcript shown in Figure 1C, no geminin was detected in TPA-treated HEL and K562 cells or their derivatives (Figure 2A). On the other hand, cdt1 was present in all differentiated cells, except for *escargot*-expressing HA1 cells (Figure 2A, lane 4). This could be related to a broad transcriptional regulation by this repressor rather than to the inability of these cells to undergo endoreplication, because nonendoreplicating K562 cells showed cdt1 levels similar to those found in HEL and KEB cells. Thus, it seems that the presence of cdt1 during megakaryocytic differentiation is due to stabilization of the protein, because in both TPA-treated HEL and K562 cells the corresponding transcripts were downregulated (Figure 1). It can also be noted that two forms (apparent MW of 66 and 72 kDa) of the protein could be detected. It has been described that cdt1 displays different cell cycle–dependent mobilities (Nishitani *et al.*, 2001). In particular, it was shown that both S- and M-phase related forms appeared to be retarded when compared with the main 65-kDa G1 form (Nishitani *et al.*, 2001). In our cells, the minor, slower migrating band could be assigned to the S phase form, as assessed by analysis of S- and M- arrested HeLa cell extracts (see Figure 5). Interestingly, HEL cells displayed a somewhat different pattern of cdt1 content, as the S phase band was more prominent in these cells than in K562, HA1, and KEB extracts, and actually increased in TPA-treated cell extracts. Thus, this appeared to be unique to HEL cells, and did not show any apparent relationship to the ability to endoreplicate in the presence of differentiating agent.

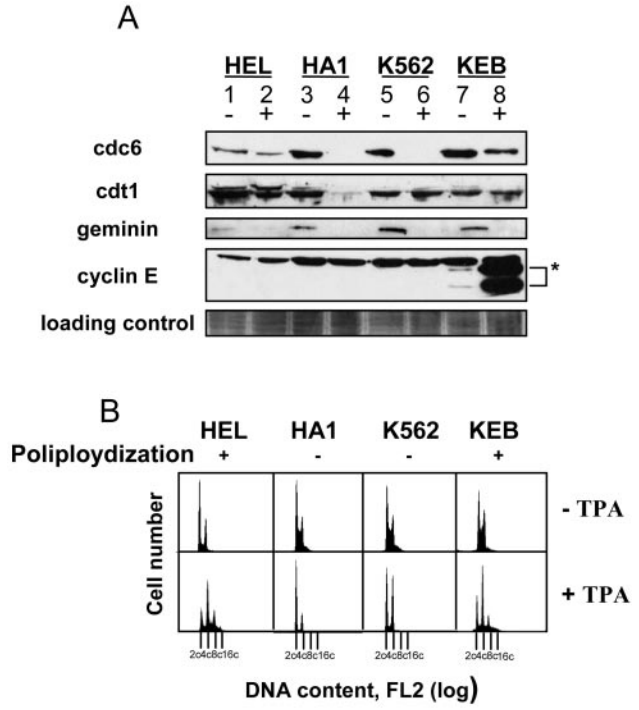

Figure 2. Cdc6 protein levels are only detected in differentiated endoreplicating megakaryoblastic cells. (A) Exponentially growing (−) and TPA-treated (+) HEL, HA1 (Dm*escargot*-expressing HEL-derived line), K562 and KEB (cyclin E-overexpressing K562-derived line) cells were collected, and whole protein extracts were obtained. Total protein, 30 μg, was subjected to SDS-PAGE and detected by Western blotting with anti-cdc6, -cdt1, -geminin, and -cyclin E antibodies. A portion of total protein staining of transferred gel is shown as a loading control. Asterisk marks the faster migrating cyclin E bands, which correspond to the protein overexpressed in KEB cells. (B) DNA content pattern of these cells is shown as analyzed by flow cytometry. Vertical axis, relative number of cells; horizontal axis, relative red fluorescence (FL2) in a logarithmic scale, indicating DNA content per cell. The positions of peaks representing cells with a DNA content equal to 2C, 4C, 8C, and 16C are indicated. The ability of these cells to achieve (+), or not (−), polyploid DNA content is indicated.

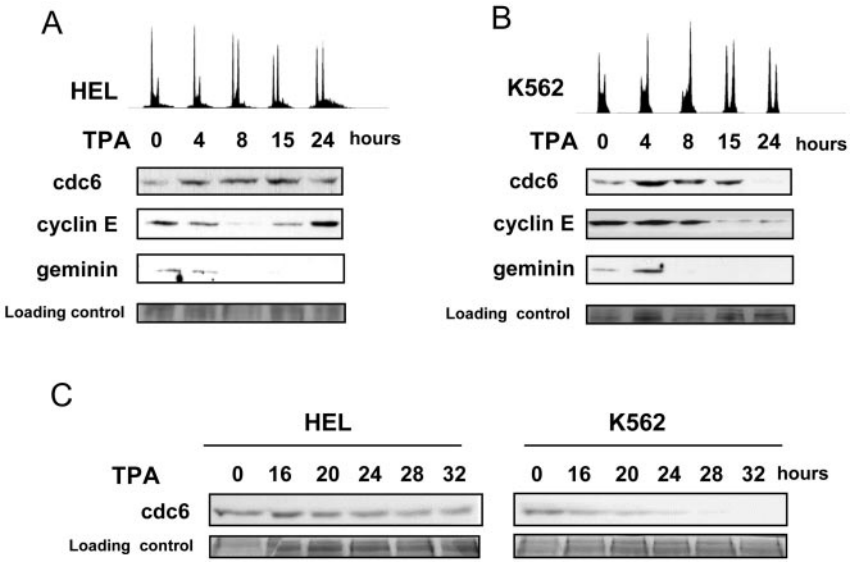

Figure 3. Cdc6 stabilization is concomitant with the establishment of the first endoreplication cycle. Whole protein extracts from HEL (A) and K562 (B) cells were obtained before treatment (0 h) or at 4, 8, 15, or 24 h after differentiation induction. Total protein, 30 μg, was subjected to SDS-PAGE and detected by Western blotting with anti-cdc6, -geminin, and -cyclin E antibodies. A portion of the total protein staining of transferred gel is shown as a loading control. Flow cytometry analysis of HEL and K562 DNA content is shown. (C) An identical experiment performed with extracts obtained form HEL and K562 cells at the indicated times after TPA treatment.

These results suggest that megakaryocytic endomitosis is characterized by the maintenance of cdc6 and cdt1 protein expression, together with the downregulation of geminin. However, only the presence/absence of cdc6 can be related to the ability/inability of megakaryoblastic cells to undergo endomitosis, because geminin and cdt1 did not show a differential regulation pattern in endoreplicating and nonendoreplicating cells.

Cdc6 Stabilization Is Concomitant to the Establishment of the First Endoreplication Cycle, whereas Geminin Downregulation Takes Place Earlier

A high proportion of polyploid HEL or KEB cells is effectively detected from 2 days after TPA treatment onward, although changes in the expression pattern of regulatory cell cycle factors take place much earlier after treatment with the differentiation signal (Garcia *et al.*, 2000; Ballester *et al.*, 2001). We thus asked whether cdc6 and geminin expression could be subjected to short-term regulation in endoreplicating versus nonendoreplicating cells. Time-course Western blot analysis was then performed at 4, 8, 15, and 24 h after TPA treatment of HEL and K562 cells.

As can be seen in Figure 3, cdc6 was slightly upregulated in both HEL and K562 cells during the first 4 h of differentiation. However, from this time point onward, the protein levels were found to be differentially regulated. In K562 cells, cdc6 levels diminished at 8- and 15-h time points and became nearly undetectable 24 h after TPA treatment. In contrast, cdc6 protein was still present in HEL cells at the 15-h time point and also 24 h after TPA treatment, at levels similar to those found after longer treatments (Figure 2A). Such different behavior between the cell lines was more apparent in an extended time-course analysis of the timing of cdc6 downregulation in K562 cells, i.e., from 15 to 24 h. As shown in Figure 3C, cdc6 levels gradually diminished in K562 cells from 16 h up to 32 h after TPA treatment but remained unchanged in HEL cells during this time. It is interesting to note that from 8 h onward, a significant number of HEL cells started to show DNA contents equal to or higher than 4N, thus suggesting the starting point of endomitotic cycles. Cdc6 levels and the onset of endoreplication followed a similar pattern in TPA-treated KEB cells (unpublished data). At this time point (8 h), cyclin E appeared also to be downregulated in HEL cells, although its expression was restored to levels similar to those found in exponentially growing cells as cells proceeded to endoreplication.

On the other hand, geminin levels remained unchanged up to 4 h in both HEL and K562 cells but were dramatically downregulated after this time, independently of the ability of cells to undergo endomitotic cycles. It can then be noted that geminin was absent at the time HEL cells started to undergo endomitotic cycles. Cdt1 levels did not change during the time analyzed in HEL nor K562 cells (unpublished data).

Altogether, these results suggest that the differences in cdc6 expression observed between endoreplicating and nonendoreplicating cells 2 days after differentiation induction are detectable as early as 8 h after TPA treatment. They also indicate that this time point broadly parallels the time of establishment of endomitotic cycles and that geminin is already absent at this point.

Cdc6 Is Present in Nuclear Compartment Only in Endoreplicating Cells

We then wanted to determine cdc6 subcellular localization in cells undergoing endoreplication cycles. Western blot analysis was performed on nuclear and cytoplasmic protein extracts obtained from HEL, HA1, K562, and KEB cells untreated and treated with TPA for 48 h. Cell fractionation was assessed by immunodetection of cytoplasmic (IκBα) and nuclear (PCNA) proteins (Figure 4A). As expected, cdc6 levels were barely detected in nuclear and cytoplasmic extracts of nonendoreplicating TPA-treated K562 and HA1 cells (Figure 4A). In contrast, nuclear cdc6 was maintained in

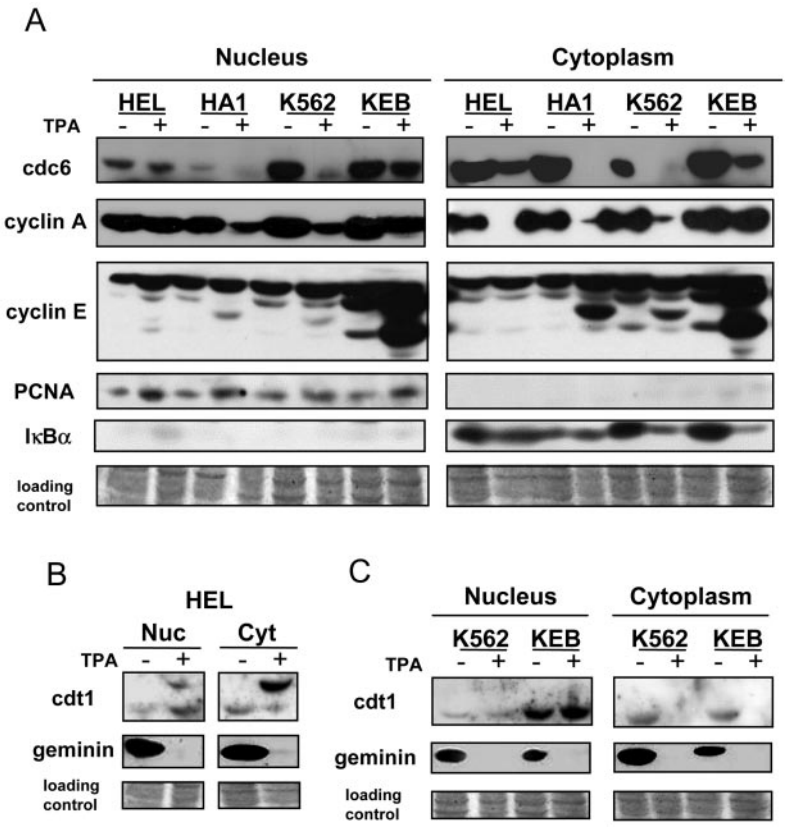

Figure 4. Nuclear cdc6 levels are maintained in endoreplicating megakaryoblastic cells. (A) Exponentially growing (−) and 48-h TPA-treated (+) HEL, HA1, K562, and KEB cells were collected. Protein from nuclear and cytoplasmic fractions was extracted. Nuclear or cytoplasmic total protein, 100 μg, was subjected to SDS-PAGE and detected by Western blotting with anti-cdc6, -cyclin A, and -cyclin E antibodies. Blots were stripped and reincubated with anti-PCNA and −I 75 Bα antibodies to assess the purity of subcellular fractions. (B) Exponentially growing (−) or TPA-treated (+) HEL cells were collected. Cdt1 and geminin expression was detected by Western blot in both nuclear (Nuc) and cytoplasmatic (Cyt) fractions. (C) Nuclear and cytoplasmic protein extracts from exponentially growing (−) or differentiated (+) K562 and KEB cells were analyzed by Western blotting with anti-cdt1 and -geminin antibodies. Portions of the total protein staining of transferred gels are shown as loading controls.

endoreplicating HEL and KEB cells, whereas the levels of cytoplasmic protein were concomitantly downregulated. Also, the levels of nuclear cyclins E and A were higher in endoreplicating HEL and KEB extracts than in TPA-treated K562 and HA1 cells.

When cdt1 distribution was analyzed, we observed that a large proportion of the protein remained nuclear in TPA-treated HEL cells (Figure 4B). Similarly to what we found when total extracts were used (see Figure 1A), the slower migrating form of the protein was more prominent in differentiated than in exponentially growing cells. Also, it could be seen that this form was mainly located in the cytoplasmic fraction of TPA-treated cells. Although KEB cells showed an overall higher nuclear cdt1 content than K562 cells, the relative levels were unchanged after TPA treatment, and the protein remained nuclear in both differentiated cells. No slower migrating form could be detected in these cells. As expected, no geminin was present in the extracts from differentiated HEL, K562, and KEB cells.

To characterize the extra cdt1 band detected in TPA-treated HEL cells, we performed Western blot analysis of total protein extracts from cells exponentially growing and after 48 and 96 h of TPA treatment. As seen in Figure 5A, the slower migrating form accumulated over the experiment time course and was the major form detected after 4 d of differentiation, when cells are still undergoing polyploidization (see DNA content analysis). This result was also obtained when a different polyclonal antibody was tested (Figure 5A, Ab3 blot). This band was identified as the form most abundantly expressed in S-phase cells, because it comigrated with the major band present in extracts from thymidine-treated, S-phase synchronized HeLa cells (Figure 5B, lanes 1 and 2). It also appeared to be clearly distinguishable from the mitotic, even-slower migrating form seen in nocodazol-treated HeLa extracts (Figure 5B, lane 3). To determine whether this mobility shift was due to a different phosphorylation state, TPA-treated HEL extracts were incubated with CIAP before SDS-PAGE electrophoresis. As can be seen in Figure 5C, no change in the pattern of cdt1 bands could be detected after enzymatic treatment. As an unrelated phosphoprotein, i.e., heat shock factor 1 (HSF-1), responded to CIAP treatment, this result suggests that the mobility shift of the cdt1 S-phase–related form was not due to phosphorylation. It cannot be discarded, however, that putative phosphorylated sites are not accessible to this enzyme or that they could preferentially be recognized by other phosphatases.

Altogether, these results show that both cdt1 and cdc6 remain nuclear in HEL and KEB cells undergoing endoreplication. They also indicate that the only apparent difference between K562 cells and KEB cells, their cyclin E–overexpressing derivatives, lies in a differential regulation of cdc6 expression.

Cdc6 Is Stabilized in Endoreplicating K562 Cells with High Levels of Cyclin E

To investigate if cyclin E overexpression in KEB cells was determining the maintenance of cdc6 at RNA or protein

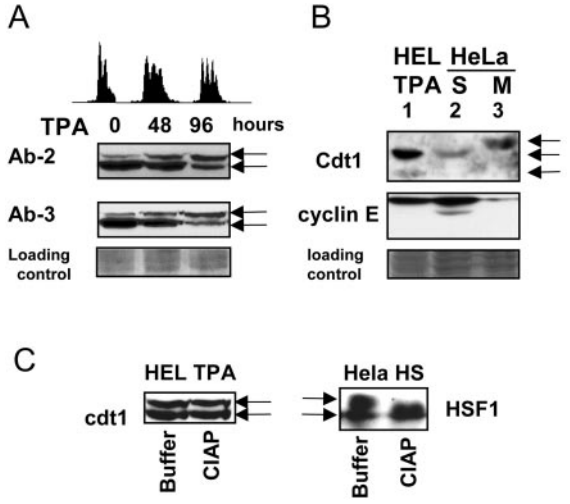

Figure 5. A slower migrating form of cdt1 is stabilized in endoreplicating HEL cells. Total protein extracts from HEL cells untreated (0 h) or treated with TPA for 48 and 96 h were subjected to SDS-PAGE and analyzed by Western blotting for cdt1 expression with two independent anti-cdt1 polyclonal antibodies (Ab2 and Ab3). (B) Total protein extracts from S- and M-phase synchronized HeLa cells (lanes 2 and 3, respectively) and cytoplasmic protein extract form HEL cells treated with TPA for 96 h were analyzed with anti-cdt1 polyclonal antibody Ab2. (C) Total protein extract from TPA-treated HEL cells (HEL TPA) was incubated with calf intestine alkaline phosphatase (CIAP) or with buffer only (Buffer) for 15 min at 30°C. HeLa cells subjected to heat-shock at 42°C for 1 h (HeLa HS) were used to detect heat shock factor 1 (HSF-1) phosphorylation. Phosphatase reaction was performed in total protein extracts form these cells as a control of CIAP activity. Portions of the total protein staining of transferred gels are shown as loading controls. Arrows indicate the different migrating cdt1 forms.

level, we first examined the levels of cdc6 transcripts in these K562-derived cells. As shown in Figure 6A, the levels of cdc6 RNA were not upregulated by cyclin E overexpression, as they slightly diminished in TPA-treated KEB cells. Then, in order to further assess whether cyclin E overexpression was influencing protein stability, we asked how an exogenous, CMV promoter-driven tagged form of cdc6 would be affected in these cells.

Both KEB and parental K562 cells were transiently transfected with a myc-tagged human cdc6 protein encoding plasmid (pMT-cdc6) or the empty vector (pMT), and either treated with TPA for 48 h or left to grow exponentially over the same time period. Whole cell extracts were obtained and analyzed by Western blotting with anticdc6 (results shown in Figure 6) and myc-tag 9E10 epitope antibodies, to confirm the identity of the exogenous, myc-tagged protein (unpublished data). Surprisingly, no ectopic cdc6 could be seen in exponentially growing pMT-cdc6 transfected K562 cells (Figure 6B, lane 6), and myc-tagged cdc6 protein could be faintly detected in these cells only over a long film exposure, and only after TPA treatment (see overexposed blot in the Figure 6). In contrast, myc-tagged protein was present at high levels in TPA-treated KEB cells (Figure 6B, lane 12) and to a much lesser extent in exponentially growing cells, as can be seen in the overexposed blot. Thus, the exogenous protein reached higher expression levels after TPA treatment in cyclin E–overexpressing cells. To determine whether the stabilized myc-tagged protein was located in the nuclear compartment, KEB cells transfected with pMT-cdc6 or pMT were cytospun, incubated with the anti-myc 9E10 epitope antibody or an isotype control, and analyzed by confocal microscopy. It could be seen that only pMT-cdc6–transfected cells were labeled with the anti-myc antibody. Whereas exponentially growing cells were faintly positive (unpublished data), myc-tagged cdc6 was clearly detected in the nuclei of a higher percentage of TPA-treated KEB cells, as shown in a representative projection of the captured images (Figure 6C). These results suggest that the stabilization of constitutively expressed myc-tagged cdc6 protein is related to the high cyclin E levels reached in TPA-treated KEB cells.

To confirm this hypothesis, K562 cells were transiently cotransfected with an expression vector containing cyclin E cDNA (pcDNA3-cycE) and pMT-cdc6, pcDNA3-cycE and empty pMT or empty pcDNA3 and pMT-cdc6. We have previously shown that transient expression of cyclin E is able to drive K562 cells into endoreplication cycles after TPA treatment (Garcia *et al.*, 2000). When Western blot analysis was carried out to assess cdc6 expression, it could be observed that myc-tagged cdc6 levels significantly increased in the TPA-treated cells, thus overexpressing cyclin E (Figure 6D, compare lanes 6 and 8). In fact, in nonoverexposed blots, the only fraction in which the protein could be detected corresponded to TPA-treated cotransfected cells (unpublished data). Interestingly, when cells were transfected with a nonfunctional cdc6 mutant form that is unphosphorylatable by cyclin-cdk complexes (Herbig *et al.*, 2000), it could be seen that this cyclin E–related stabilization did occur with much lower efficiency (Figure 6E, compare lanes 4 and 5).

Altogether, these results indicate that overexpression of cyclin E in K562 cells results in the maintenance of cdc6, mainly through the stabilization of the nuclear protein and that this requires intact cdk phosphorylation sites. This suggests that endoreplication in KEB cells is due to the accumulation of both functional cyclin E and cdc6.

Cdc6 Is Able to Drive HEL Cells into an Endoreplication Cycle

We next asked whether cdc6 could have a direct role in the establishment of new rounds of DNA synthesis in HEL cells. For this purpose, HEL cells were transiently transfected with a retroviral bicistronic vector containing cdc6 and a truncated form of neural growth factor receptor (Δ-NGFR) cDNAs downstream of an *IRES* sequence that allows the translation of both proteins from a single transcript (Abad *et al.*, 2002). The expression of the bicistron was under CMV promoter control. Because Δ-NGFR is directed to the cell surface but lacks any downstream activity, it serves as a means of isolating transfected cells by use of immunomagnetic devices.

HEL cells were transfected with the vector containing cdc6-*IRES*-Δ-NGFR or *IRES*-Δ-NGFR only (hereafter referred as to "cdc6" and "control," respectively) and then stimulated with TPA for 48 h or allowed to grow exponentially in culture medium for the same time period. Before proceeding to their separation, their DNA content was measured and found to be identical in cdc6- and control-transfected cells (Figure 7, whole population). Cells were then

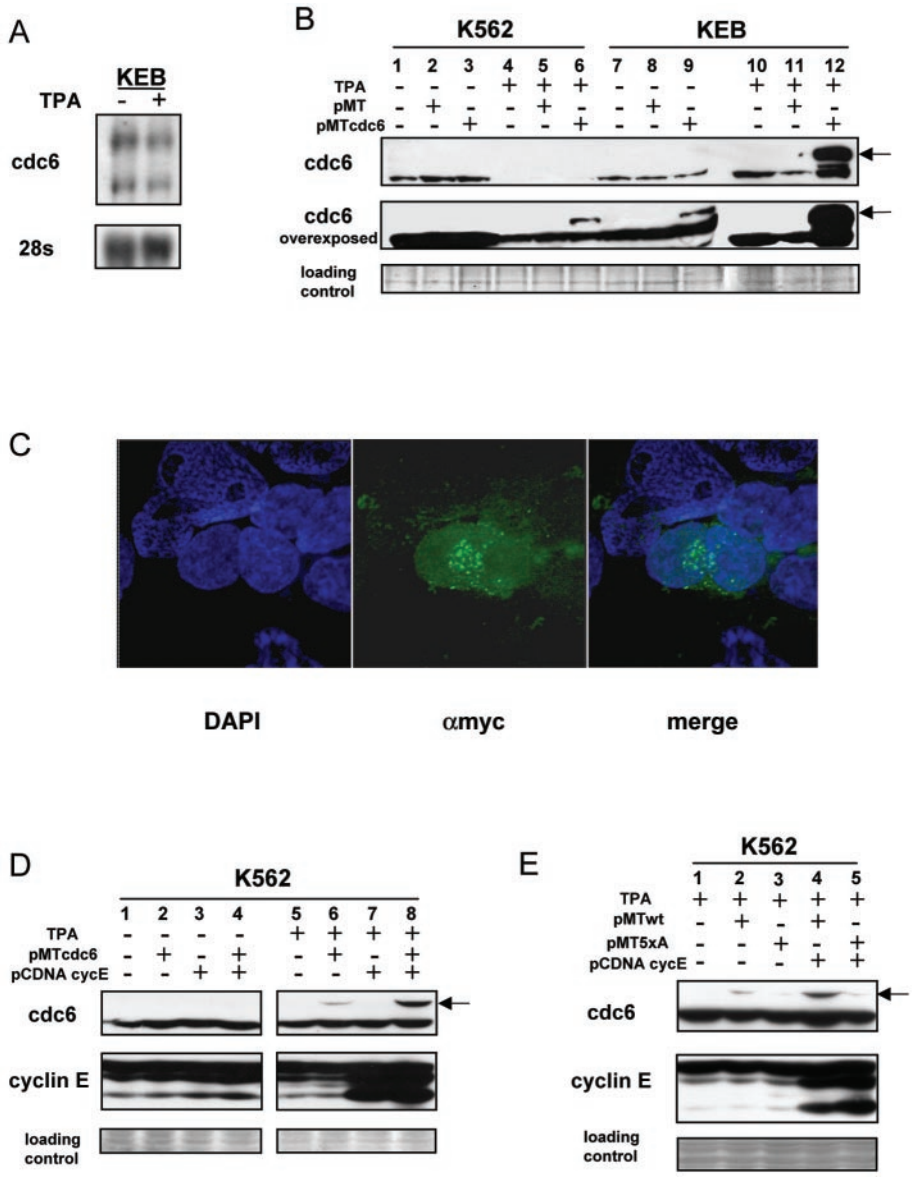

Figure 6. Cdc6 is stabilized in K562 cells with high levels of cyclin E. (A) KEB cells were cultured for 48 h in the absence (−) or the presence (+) of TPA and total RNA was extracted. RNA, 20 μg, was analyzed by Northern blot with full-length cdc6 cDNA as a probe. The same blot was incubated with a probe recognizing 28S ribosomic RNA and is shown as a loading control. (B) K562 and KEB cells were transiently transfected with either pMT or pMTcdc6 plasmids. Nontransfected, pMT-transfected, and pMTcdc6-transfected cells were allowed to grow exponentially (−) or treated with TPA for 48 h (+). Whole protein fractions were obtained, and Western blot analysis with anticdc6 antibody was performed. An overexposed film is also shown to stress the limited expression of myc-tagged cdc6. (C) pMTcdc6-transfected KEB cells, 2×10^4, were cytospun, fixed with formaldehyde, permeabilized with TX100, and incubated sequentially with anti-myc 9E10 and Alexa Fluor 488–conjugated anti-mouse Ig antibodies. Nuclei were counterstained with DAPI. The figure shows the maximal projection (blue, green, and merge) of an image at 1024 × 1024 resolution. (D) K562 cells were transiently cotransfected with pMTcdc6 and pcDNA-cycE or the corresponding empty plasmids. Either exponentially growing (−) or TPA-treated (+) cells were analyzed by Western blot using anti-cdc6 and -cyclin E antibodies. (E) Western blot analysis with anti-cdc6 and -cyclin E antibodies of TPA-treated pMT-, pMT-cdc6 wild-type (pMTwt)- or pMT- cdc6 5xA mutant (pMT5xA)-transfected cells, and cells cotransfected with pMTwt or pMT5xA plasmids, together with pCDNA-cyclin E are shown. Portions of the total protein staining of transferred gels are shown as loading controls. Both D and E show overexposed blots.

labeled with the anti-NGFR mAb. Δ-NGFR–expressing cells (transfected) were then separated from Δ-NGFR–nonexpressing (nontransfected) cells by incubation with anti-mouse Ig coupled to magnetic beads. Both populations (NGFR-positive; NGFR-negative) were collected and their DNA content analyzed by flow cytometry. As can be seen in Figure 7, all treated cells responded to TPA as expected and underwent at least one endoreplication cycle. This was independent of whether they were expressing the ectopic proteins (NGFR-positive cells) or not (NGFR-negative cells) and in the case of NGFR-positive cells, whether they contained cdc6 or vector only. No apparent effect of cdc6 overexpression could be detected, in terms of further potentiation of TPA-driven endoreplication. However, exponentially growing HEL cells that had been transfected with cdc6 presented a nearly identical DNA content pattern to that of the TPA-treated cells. This phenotype (endoreplication in the absence of a differentiation stimulus) was only detected in cdc6-expressing cells, because both the NGFR-negative cdc6-transfected cells and NGFR-positive cells transfected with vector only had the typical DNA content pattern corresponding to exponentially growing cells.

These results indicate that ectopic expression of cdc6 is able to promote by itself the endoreplication in HEL cells to a comparable degree to that reached after TPA treatment

DISCUSSION

Megakaryocytic endomitosis represents a valuable system for the study of how mammalian cells are able to overcome the strict rereplication restrictions imposed to most somatic

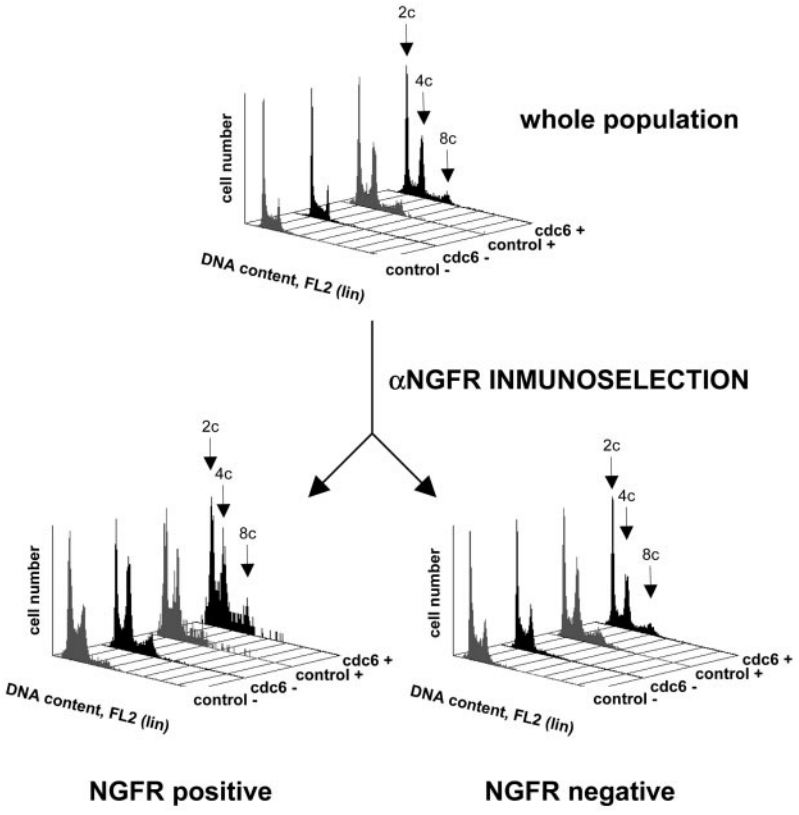

Figure 7. Cdc6 is able to drive proliferating HEL cells into the endoreplication cycle. HEL cells were transiently transfected with pLZR-CMV-IRES-ΔNGFR empty vector (control) or pLZR-CMV-cdc6-IRES-ΔNGFR construct (cdc6) and allowed to grow exponentially (−) or treated with TPA for 48 h (+). DNA content of whole population, NGFR-positive, and NGFR-negative immunoselected cells was analyzed by flow cytometry. Arrows indicate the position of 2C, 4C, and 8C DNA content cell peaks. The histograms shown are representative of four independent experiments.

cells. By comparing two megakaryoblastic cell lines that both undergo megakaryocytic differentiation upon TPA treatment but display different abilities to become polyploid, we have found that the basic DNA replication initiation machinery is differentially regulated during endoreplication. We have shown that the differentiation stimulus determines geminin downregulation, together with cdt1 stabilization. Also, cdc6 is stabilized only in endoreplicating cells, and cyclin E may play an important role in such stabilization. Finally, we show that ectopic cdc6 expression in megakaryoblastic cells fully determined to become polyploid provokes the entrance into endomitosis in the absence of differentiation stimuli.

Geminin Downregulation Is Concomitant with Megakaryocytic Differentiation

Geminin was first described as a potent inhibitor of DNA replication and as a neutralizing molecule during *Xenopus* development (Kroll *et al.*, 1998; McGarry and Kirschner, 1998). Interestingly, recent reports have independently shown that geminin avidly binds to cdt1 (Wohlschlegel *et al.*, 2000; Tada *et al.*, 2001). Indeed, it has been proposed to be one of the mechanisms that ensures the silencing of already fired origins until completion of the entire cycle (Lygerou and Nurse, 2000). Geminin is expressed from S phase to the onset of mitosis, when it becomes degraded via the APC/C system (McGarry and Kirschner, 1998). Therefore, one could expect that this mechanism would also be operative in megakaryocytic endomitosis, as cells transit normally from metaphase to anaphase, only skipping subsequent mitotic steps (Nagata *et al.*, 1997; Vitrat *et al.*, 1998). However, our results show that geminin is significantly downregulated in megakaryocytic cells, soon after being stimulated to differentiate, and independently of whether the cells further establish endoreplication cycles.

As geminin has been proposed to play an important role in determining neural cell fate (Kroll *et al.*, 1998; Quinn *et al.*, 2001), it has been hypothesized that an increase of geminin expression could help differentiating cells to withdraw from the cell cycle (Madine and Laskey, 2001). High levels of geminin would thus be expected upon any differentiation stimulus. Obviously, this is not the case in megakaryocytes. The simplest interpretation is that the differentiation-driven downregulation of geminin would be a necessary event for allowing these particular cells to complete their differentiation program, ultimately and uniquely necessitating an escape from rereplication control. Genetic experiments in *Drosophila* embryos and cultured cells have shown that silencing of geminin provokes actual overreplication (Quinn *et al.*, 2001; Mihaylov *et al.*, 2002). It could then be inferred that geminin downregulation is a requirement for cells to endoreplicate. However, *Drosophila* geminin appears to be normally expressed in endoreplicating tissues of the gut and in adult ovarian nurse and follicle cells (Quinn *et al.*, 2001). One possible explanation for this apparent discrepancy between megakaryocytes and insect cells could lie on the fundamental differences between the endocycles of these *Drosophila* cells and megakaryocytic endoreplication. As mentioned

above, the latter actually comprises early mitotic stages (Nagata *et al.*, 1997; Vitrat *et al.*, 1998), whereas the former skip the G2/M transition (Lilly and Spradling, 1996). Hence, it would be interesting to explore whether the silencing of geminin has any additional meaning in the context of the abortive mitosis that takes place in megakaryocytes. In this respect, it has been proposed that geminin could have a role in anaphase, at some stage after cyclin B degradation (Quinn *et al.*, 2001). Cyclin B degradation also occurs in endomitotic cycles, so it is tempting to speculate that at this point the absence of geminin could at least be one of the events that prevent completion of anaphase in differentiated megakaryocytes.

Cdc6 Is Specifically Maintained in Differentiated Megakaryoblastic Cells that Become Polyploid

Our data clearly show that the main difference between endoreplicating and nonendoreplicating megakaryocytic cells lies in the presence of cdc6, because in nonendoreplicating cells its expression is severely compromised. In an unrelated cell system such as *Arabidopsis* endoreplicating cells, cdc6 expression has also been found to be maintained (Castellano *et al.*, 2001; Ramos *et al.*, 2001). In one of these reports, the authors propose that the maintenance of cdc6 levels in dark-grown hypocotolyl cells is assured both by active gene expression and protein stabilization (Castellano *et al.*, 2001). Our data suggest that in megakaryocytes these mechanisms can also account for the continuous presence of the protein. Thus, in endoreplicating TPA-treated HEL cells, the RNA levels of cdc6 are maintained and even increased long after endoreplication cycles have been established, whereas they are downregulated in K562 cells. Additionally, our data strongly suggest that stabilization of the protein can be a second mechanism by which cdc6 is maintained in endoreplicating cells. First, not only are cdc6 levels maintained in HEL; a particularly high proportion of the protein remains nuclear. Second, endoreplication of cyclin E–overexpressing K562 cells results in the presence of the nuclear protein, with no major stimulation of cdc6 transcripts levels. Thus, it can be suggested that megakaryocytic endoreplication involves specific regulation of both transcription and stabilization of the protein.

Cyclin E Is Responsible for cdc6 Stabilization in Endoreplicating Cells

One interesting observation in this article is that cyclin E appears to contribute to cdc6 stabilization during megakaryocytic endoreplication. Cdc6 protein is detected in TPA-treated K562 cells that become competent to undergo polyploidization by stably overexpressing cyclin E (KEB cell line). This could be interpreted as a nonspecific effect of cyclin E induction of the expression of G1/S downstream regulators, i.e., cyclin A (Zerfass-Thome *et al.*, 1997; Garcia *et al.*, 2000). Thus, it could simply be due to an increased transcription rate, as a consequence of cells being able to proceed through G1 and on to S, because cdc6 expression seems to be controlled through E2F (Hateboer *et al.*, 1998; Ohtani *et al.*, 1998; Yan *et al.*, 1998). However, our results suggest a direct role for cyclin E in cdc6 protein stabilization. First, cdc6 steady-state RNA decreased in differentiated KEB and did not reach the levels present in endoreplicating HEL cells, indicating that overexpressed cyclin E is not inducing cdc6 expression. Second, the stabilization of total cdc6 protein paralleled the establishment of endoreplication and cyclin E stabilization, and its preferential nuclear localization is coincident with that of cyclin E (this article and Garcia *et al.*, 2000). Third, an exogenous myc-tagged cdc6 protein, whose expression was not dependent on cell cycle progression, could only be detected in TPA-treated KEB cells and also in TPA-treated, cyclin E-transiently transfected K562 cells.

To our knowledge, this is the first time that such a relationship between cyclin E and cdc6 has been observed. It is noteworthy that the pattern of expression and cellular localization of both proteins are strikingly coincident in mitotic, and also in endomitotic, cycles (this article; Koff *et al.*, 1991; Dulic *et al.*, 1992; Garcia and Cales, 1996; Garcia *et al.*, 2000). Also, cdc6 and cyclin E appear to have a synergistic effect on inducing S-phase entry of cotransfected cells (Hateboer *et al.*, 1998) as well as in a cell-free system (Coverley *et al.*, 2002). A direct role for cdc6 as a chromatin anchor for the cyclin E-cdk2 complex has even been proposed (Furstenthal *et al.*, 2001). It can thus be speculated that both proteins stably interact whenever they coincide spatiotemporally. In a regular mitotic cycle, cyclin E and cdc6 levels peak at the G1/S transition and coincidentally increase in the nucleus around that time. However, shortly after S phase onset, cyclin E is proteolysed and only starts to accumulate after completion of mitosis. So, if an interaction between cdc6 and cyclin E does occur, it can only take place within a narrow time window in proliferating cells. In contrast, in megakaryocytic cells undergoing endomitosis, high levels of cyclin E persist throughout the process, probably reflecting a specific regulatory mechanism (Garcia and Cales, 1996; Datta *et al.*, 1998; Garcia *et al.*, 2000). It could be argued that cyclin E permanence in the nucleus after completion of endomitotic S phase could in turn influence cdc6 stabilization. A similar situation, e.g., polyploidization related to stabilization of cyclin E beyond S-phase, has been observed in another mammalian cell type. In $skp2^{-/-}$ mice hepatocytes, the silencing of this component of the ubiquitin-ligase complex SCF determines cyclin E stabilization and the potentiation of endoreplication of these cells not only in vitro (Nakayama *et al.*, 2000), but also "in vivo" (Minamishima and Nakayama, 2002). It would be interesting to explore whether cdc6 is equally stabilized in these endoreplicating hepatocytes.

Cdc6 Is Able to Drive Proliferating HEL Cells into Endomitotic Cycles

A striking observation of this article is that transient expression of cdc6 is able to drive endoreplication in proliferating HEL cells. This effect is reminiscent of earlier reports on other cell types such as *Schizosaccharomyces pombe* (Nishitani and Nurse, 1995) and more recently in *Arabidopsis thaliana* (Castellano *et al.*, 2001). However, the mechanism by which high levels of cdc6 are able to trigger the entrance into extra S-phases has not been elucidated to date. Interestingly, *Saccharomyces cerevisiae* cdc6 inhibits mitotic cdk activity, thus delaying the progression into mitosis (Calzada *et al.*, 2001; Weinreich *et al.*, 2001). If this were a conserved action of cdc6, it would be possible that in HEL and *A. thaliana* cells a cdc6-mediated delay in G2/M transition, together with its licensing function, would facilitate origin relicensing and hence the entrance into endoreplication cycles. In fact, some megakaryoblastic cells become polyploid when mitosis progression is inhibited by nocodazol treatment (van der

Loo *et al.*, 1993). However, cdc6 overexpression was not able on its own to drive K562 nor KEB cells into endoreplication (R.B., N.V., and C.C., unpublished results). This has led us to think that cdc6 mitotic inhibition cannot be the sole explanation of how cdc6 is driving proliferating HEL cells into polyploidization. Actually, cdc6 overexpression does not provoke endoreplication in *S. cerevisiae* (Drury *et al.*, 1997) or other mammalian cells (Ohtani *et al.*, 1998; Petersen *et al.*, 2000). Our interpretation is that only cells determined to establish endoreplication cycles will respond to cdc6 overexpression. This would be the case for HEL cells, in contrast with K562 cells, which correspond to cells with a more undifferentiated phenotype. It can be speculated that in cells developmentally prone to endoreplicate, i.e., HEL or physiologically endoreplicating *A. thaliana* cells, additional factors might be present that synergize with overexpressed cdc6. Intriguingly, cdt1 appears to be differentially regulated in HEL cells. A stabilized S-phase form, expressed at low levels in proliferating cells, was found to be uniquely upregulated during HEL endoreplication. Furthermore, this form was more prominent in exponentially growing HEL than in K562 or KEB cells. We could hypothesize that this differential feature reflects the readiness of HEL cells to endoreplicate. If this were the case, it could be that a particularly high proportion of both cdc6 and S-phase stabilized cdt1 coincide in cdc6-transfected HEL cells. This synergy between cdt1 and cdc6 would be responsible for origin licensing and entrance into extra S phases. This situation is reminiscent to the rereplication that takes place in *Xenopus* sperm nuclei when cdk activity and geminin are blocked in metaphase extracts (Tada *et al.*, 1999) or in *S. pombe,* when both cdc6 and cdt1 are overexpressed in G2 phase (Yanow *et al.*, 2001).

In conclusion, we have shown that megakaryocytic differentiation determines the downregulation of geminin but that endoreplication is only allowed when both cdc6 and cdt1 expression are stabilized

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