Oncogene (2000) 19, 1820–1833

www.nature.com/onc

Ectopic expression of cyclin E allows non-endomitotic megakaryoblastic K562 cells to establish re-replication cycles

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Megakaryocytes become polyploid by entering a truncated cell cycle, consisting of alternate S phases and abortive mitoses. We have investigated the regulation of the G1/S transition by comparing two megakaryoblastic cell lines, HEL and K562, which respectively do or do not become polyploid in response to phorbol esters. A pronounced downregulation of cyclin A, and to a lesser extent of cyclin E, occurred in K562 cells during the first 24 h after TPA treatment, in contrast with re-replicating HEL cells, in which both cyclins were present in individual G2/M cells. Transactivation experiments suggested that the absence of cyclin A in differentiated K562 cells could be due to a TPA-mediated inhibition of its transcription. To investigate the potential role of cyclin E in the establishment of re-replication cycles, we isolated K562 clones constitutively expressing cyclin E. The resulting clones, and also K562 cells transiently expressing cyclin E, entered re-replication cycles when treated with TPA. The transcriptional activity of the cyclin A promoter was not inhibited after TPA treatment, and although the levels of cyclin A fluctuated during further re-replication cycles, they never decreased below S phase levels. We conclude that the presence of cyclin E in megakaryoblastic G2/M cells determines cyclin A expression and allows the entrance into an extra S phase. *Oncogene* (2000) **19,** 1820–1833.

Keywords: cyclins; cell cycle; re-replication; megakaryocytic differentiation; transcriptional regulation

Introduction

Megakaryocytes arise from marrow precursors that proliferate and progressively acquire a distinct lineage phenotype, leading to mature cells that ultimately produce circulating platelets. Uniquely within this blood cell lineage, an as yet unknown external signal causes differentiating megakaryoblasts to stop proliferating and to enter a peculiar cell cycle consisting of repeated rounds of DNA synthesis without concomitant cell division. The resulting polyploid megakaryocyte has a dramatically increased cytoplasmic volume and nuclear size, and actively synthesizes all platelet components. Finally, and through a poorly defined mechanism, functional platelets are shed into the bloodstream. Since the ploidy distribution of marrow megakaryocytes is affected in certain patients with thrombocytopenia (Tomer *et al*., 1989), it appears that an efficient release of platelets is related to establishment of re-replication cycles within differentiating megakaryoblasts.

In addition to mammalian megakaryocytes, polyploidization occurs in a number of eukaryotic cells, ranging from plant endosperm to mammalian trophoblast giant cells, as part of their developmental programme, through an unusual re-replication of nuclear DNA without entry into or completion of mitosis (Grafi and Larkins, 1995; Hoffman, 1988; Smith and Orr-Weaver, 1991; Varmuza *et al*., 1988). In the majority of somatic cells, a strict regulation is needed for the proper succession of S and M phases so that daughter cell ploidy is maintained. Early morphological studies showed that megakaryocytes reached metaphase but did not proceed to further mitotic phases (Ebbe, 1976). The process was called endomitosis, and referred to the replication of nuclear DNA within an intact nuclear membrane.

Studies on normally cycling cells have indicated that one or more licensing factors are implicated in the prevention of re-replication before a cell has undergone mitosis (Chong *et al*., 1995; Madine *et al*., 1995). It seems that the ultimate control of such licensing factors is exerted by the various cyclin-cdk complexes that also govern the proper transition through the cell cycle (Krude *et al*., 1997). These universal controllers of the cell cycle appear, therefore, to be good candidates for being directly involved in the control of re-replication cycles. In re-replicating maize endosperm, the G1/S complexes remain activated, while the G2/M complex is inhibited (Grafi and Larkins, 1995). One system in which re-replication has been more extensively studied is the fruit fly *Drosophila*, in which specific subsets of cells respond to spatial and temporal controls to become polyploid during oogenesis and embryonic and larval development (Duronio and O'Farrell, 1994; Edgar, 1995; Smith and Orr-Weaver, 1991). Genetic analyses have proved that cyclin E is directly implicated in the establishment of *Drosophila* endocycles (Duronio and O'Farrell, 1995; Knoblich *et al*., 1994; Lilly and Spradling, 1996; Richardson *et al*., 1993; Sauer *et al*., 1995), and that these can occur in the absence of mitotic cyclins A and B, cdc2 or *string* (*Drosophila* cdc25C), the phosphatase that ultimately activates the cyclin B-cdc2 at the G2/M transition (Lehner and O'Farrell, 1990; Smith and Orr-Weaver, 1991; Stern *et al*., 1993). Also, in mouse trophoblasts, cyclin E appears to be expressed during polyploidization, whereas expression of cyclins A and B, and cdc2 is abolished (MacAuley *et al*., 1998).

Due to the scarcity of mature megakaryocytes and the difficulty of obtaining homogenous populations of

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Received 21 April 1999; revised 17 January 2000; accepted 25 January 2000

marrow megakaryoblasts, the use of established cell lines has constituted a convenient experimental system in order to carry out consistent biochemical analysis (Hoffman, 1988). Studies of different megakaryoblastic cell lines that progress towards mature megakaryocytic forms in response to various differentiation stimuli have shown that the failure to enter or complete mitosis occurs through alterations in the G2/M regulators cyclin B (Zhang *et al*., 1996), cdc2 (Datta *et al*., 1996), cdc25C (García and Calés, 1996) or $p21^{cip1}$ (Kikuchi *et al*., 1997; Matsumura *et al*., 1997). More recently, two laboratories have focused on the mitotic events that occur during megakaryopoiesis, and have reported that cultured megakaryocytes do not complete anaphase (Nagata *et al*., 1997; Vitrat *et al*., 1998).

With respect to G1/S regulators that have been implicated in megakaryocytic endomitosis, cyclin D3 has been reported to have a role in polyploidization (Wang *et al*., 1995; Zimmet *et al*., 1997), and all cyclins D3, E and A appear to remain complexed to cdk2 during polyploidization (Datta *et al*., 1998). In our laboratory, and by making use of two megakaryoblastic cell lines, HEL and MEG01, which differentiate towards mature forms of megakaryocytes when treated with phorbol esters, we previously analysed G1/S and G2/M regulatory factors (García and Calés, 1996). We found that cyclins E and A were not downregulated and that in contrast, cdc25C mitotic phosphatase levels were severely diminished in both differentiated cell lines.

In this paper, we have studied the establishment of re-replication cycles, in particular with respect to the regulation of the G1/S transition, in order to find differences which could explain the different behaviour between re-replicating (HEL) and non re-replicating (K562) megakaryoblastic cells. Although both cell lines respond to phorbol esters in terms of phenotypic differentiation towards mature megakaryocytes, K562 cells do not enter a re-replication cycle. We have analysed G1/S cyclins levels by Western blot and flow cytometry. We have also examined the transcriptional activity of cyclin A promoter, which appears to be inhibited in response to TPA in non re-replicating cells, and could explain the absence of the protein in K562 cells. In order to investigate the potential role of cyclin E in the establishment of such cycles, we have analysed the effect of transient and constitutive expression of cyclin E in K562 cells. In both cases, cyclin E appears to confer the ability of K562 cells to enter re-replication cycles in the presence of TPA. In these cells, the cyclin A promoter is no longer inhibited by TPA and the protein is not downregulated during the first hours of TPA treatment. We conclude that the maintenance of cyclin E in G2/M cells determines cyclin A expression and the entrance into an extra S phase.

Results

Megakaryoblastic K562 cells do not enter re-replication cycles in response to TPA

Treatment of K562 cells with 10^{-8} M TPA resulted in cessation of their growth and after 48 h of treatment they showed certain features of megakarocytic differentiation, such as the appearance of the antigen CD61 and a corresponding down-regulation of the erythrocytic marker glycophorin A (Figure 1a). Analysis of the nuclear DNA content by flow cytometry showed that the majority of TPA-treated K562 cells corresponded to cells with 2C DNA content in G0/G1 phase (60%), although a significant population (26%) appeared to remain in G2 phase (Figure 1b). We could not detect an increase in the number of polyploid cells with DNA content higher than 4C, even 1 week after the treatment (data not shown). This behaviour of K562 cells is in clear contrast not only to that of non-megakaryocytic haemopoietic cell lines, such as the promonocytic U937, which respond to TPA by differentiating along the monocyte/macrophage lineage with concomitant arrest in G0/G1 (95%), but also with that of the megakaryoblastic HEL line in which more than 50% of TPA-treated cells have DNA contents of 4C or higher (Figure 1b). BrdU labelling experiments reinforced these differences, since 80–90% of HEL with 4C DNA content incorporated BrdU during a 4 h-labelling pulse (García and Calés, 1996), in contrast with K562 cells, in which only 5% of 4C cells were BrdU-positive, even after an overnight incubation (data not shown).

The fact that the TPA-treated K562 cells did not incorporate BrdU suggests that K562 cells become arrested in G1 and G2 phase when treated with phorbol esters. The results also confirm that K562 cells are able to undergo megakaryocytic differentiation, although they cannot proceed to a fully differentiated phenotype due to an impairment in the establishment of a re-replication cycle.

Re-replicating HEL and non-re-replicating K562 cells show differential expression of cell cycle regulators

In order to investigate whether the different response to TPA was paralleled by a different regulation of cell cycle proteins, we analysed the levels of factors that have a role in G1/S and G2/M transitions.

The analysis of the proteins involved in the G2/M transition (Figure 2a) revealed that in both TPA-treated HEL and K562 cells cyclin B levels remained constant, whereas cdc25C levels were down-regulated, thus in accordance with our previous results (García and Calés, 1996). The specific bands corresponding to cdc25C were determined by transient transfection with cdc25C cDNA (García and Calés, 1996 and data not shown). The only significant difference between re-replicating and non-re-replicating cells was with respect to the cdc2 protein levels. K562 extracts contained a lower amount of cdc2 protein after TPA treatment, and basically no slower migrating form of cdc2 (corresponding to phosphorylated cdc2 in G2 phase) was detected.

Western blot analysis of cyclin E protein (Figure 2b) showed that in both TPA-treated K562 and HEL cells, cyclin E levels were unchanged. However, consistent with the observation that TPA-treated K562 cells cannot proceed to S phase, analysis of cdk2 showed it to be mainly in its non-active, slowly migrating form, even though cyclin E is present (Figure 2b). In contrast, in HEL cells both forms of cdk2 were present, even after TPA-treatment (Figure 2b). Interestingly, pRb appeared to be at least partially hyper-

phosphorylated in both untreated and treated K562 and HEL cells (data not shown). This could be due to the fact that a high proportion of TPA-treated K562 remain arrested in G2 phase. It could also be assumed that at least some of the K562 are arrested in late G1 phase, consistent with the fact that cyclin E levels are maintained. The inability of these cells to enter S phase could then be due to a TPA triggered inhibitory event which would not allow K562 cells to transit from G1 to S phase. The fact that $p27^{Kip1}$ levels appeared to be higher in TPA-treated K562 than in TPA-treated HEL could explain why K562 cells cannot enter into S phase after treatment with TPA even though they maintain similar levels of cyclin E compared to TPA-treated HEL (Figure 2b). When cyclin E-associated kinase activity was assayed with recombinant GST-retinoblastoma fusion protein (GST-Rb) as substrate, we found that such activity was nearly absent in non re-replicating K562 cells whereas in TPA-treated HEL cells the kinase activity was even higher than that found in exponentially growing cells (Figure 2c). Similarly, no cdk2-associated kinase activity could be detected in TPA-treated K562 cells, in contrast with re-replicating HEL cells, in which cdk2 appeared to be active even 1 week after TPA treatment (Figure 2c, lane 3). In addition, one of the most striking differences in K562 and HEL cells was revealed when cyclin A levels were measured as shown in Figure 2d. In K562 cells, cyclin A was dramatically down-regulated after TPA treatment.

These results indicate that K562 cells respond differently to TPA treatment compared to HEL, in that they do not proceed to extra S phases, possibly because of a reduction of cdk2 activity and the absence of cyclin A expression.

Cyclin A fluctuates between G2 and S phase levels in re-replicating cells

In order to further investigate the relationship between cyclin A downregulation and lack of re-replication in K562 cells, we decided to analyse the fate of cyclin A

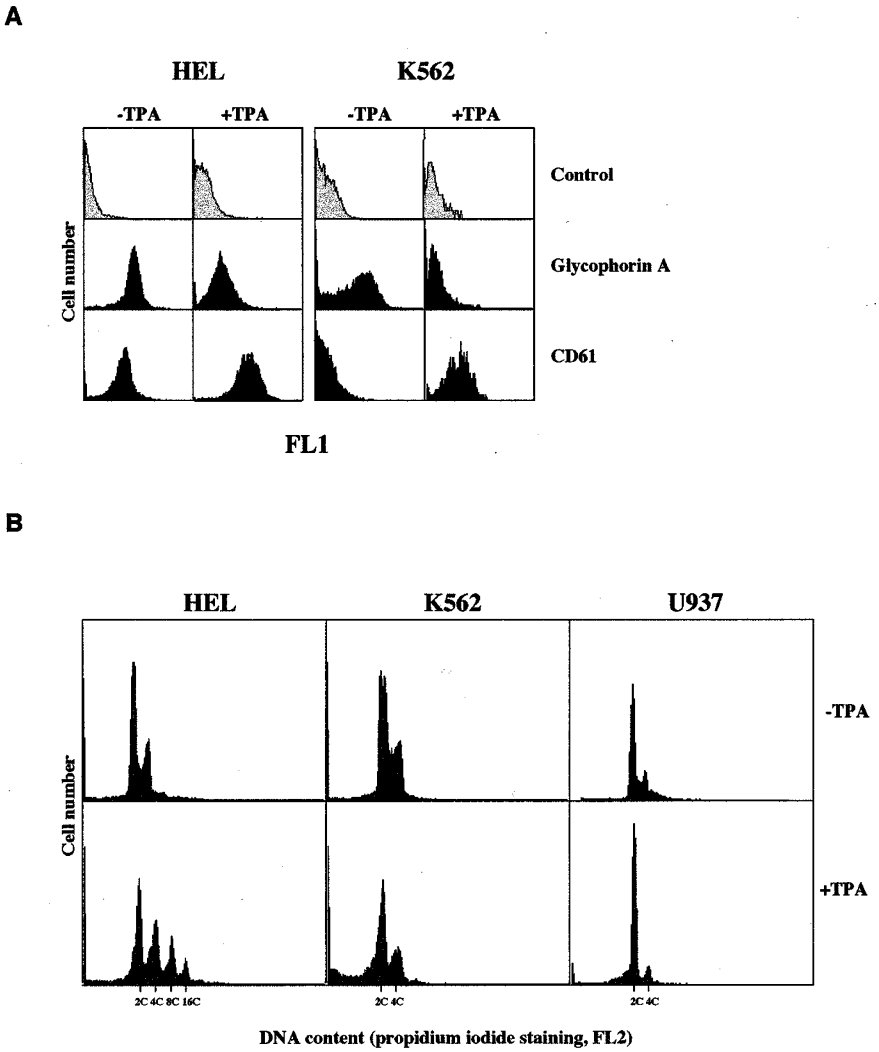

Figure 1 Effect of TPA on the megakaryocytic differentiation of K562 cells. Cells were cultured from an initial density of 1.5×10^5/ml for 96 h in the absence or presence of 10^{-8} M TPA. (**a**) Flow cytometric analysis of indirect immunofluorescence staining of TPA-treated and untreated K562 and HEL cells with anti-glycophorin A (JC159a) or anti-CD61 (Y2/51) antibodies and an isotype matched irrelevant control antibody (anti-src 327). Vertical axis – relative number of cells; Horizontal axis – relative green fluorescence (FL1) on a logarithmic scale. (**b**) Propidium iodide staining of TPA-treated and untreated K562, HEL and U937 cells. Vertical axis relative number of cells; Horizontal axis – relative red fluorescence (FL2) on a logarithmic scale indicating the DNA content per cell. The positions of peaks representing cells with a DNA content of 2, 4, 8 and 16C are indicated

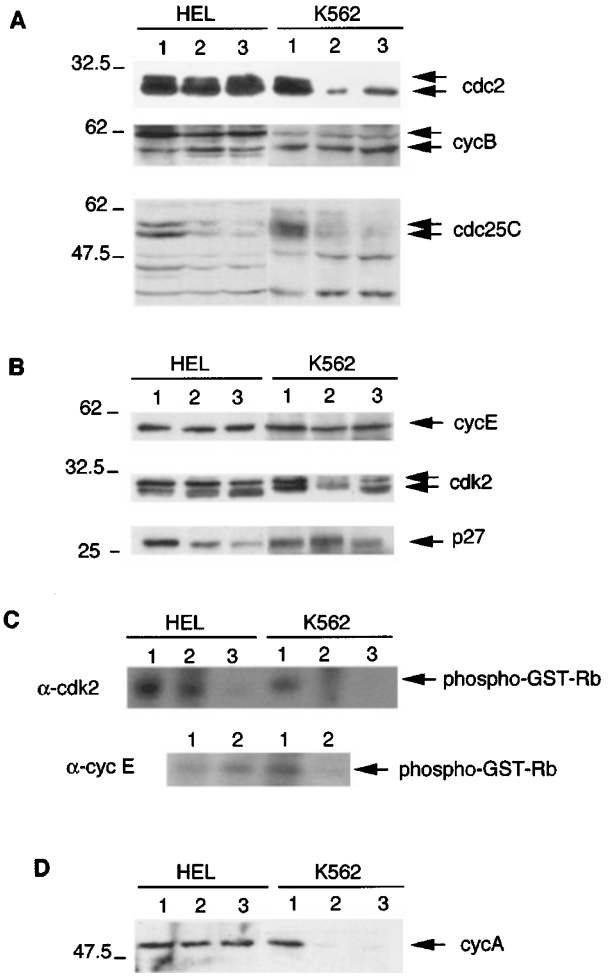

Figure 2 Comparative Western blot of cell cycle regulatory proteins and cdk2-associated kinase activity analysis in TPA-treated and untreated HEL and K562 cells. Cells were cultured in the absence or the presence of 10^{-8} M TPA. Thirty micrograms of total protein extract from cells exponentially growing, or treated with TPA for different time length were subjected to SDS–PAGE and detected by Western blot with antibodies against: (**a**) cdc2, cyclin B and cdc25; (**b**) Cyclin E, cdk2 and $p27^{kip1}$, and (**d**) cyclin A. The position of relevant molecular weight standards is shown on the left side of the blot, and the specific bands detected for each antibody are indicated by arrows on the right. (**c**) One hundred and fifty micrograms of total protein extracts were immunoprecipitated with an antibody against cdk2 or cyclin E as shown on the right. Associated kinase activity was then assayed in the presence of GST-Rb and γ-[^{32}P]ATP. Lane 1, exponentially growing cells; lane 2, cells treated with TPA for 48 h; lane 3, cells treated with TPA for 96 h (**a**, **b**, **d**) or 1 week (**c**)

in the first 24 h after TPA treatment (first putative re-replication cycle) in both K562 and HEL cells. Cyclin E content was also determined. Cells were simultaneously labelled with propidium iodide and FITC-conjugated anti-cyclin antibodies, in order to identify the nuclear DNA content of the cyclin E or A positive cells. This approach has the advantage of addressing two important points in the same experiment: (i) in which position of a mitotic or a re-replication cycle the protein is present; and (ii) how the levels of the protein fluctuate during a re-replication cycle.

Exponentially growing HEL or K562 cells showed the expected pattern of cyclin A expression: in both cases, cyclin A was absent in cells in G1 phase, and its levels steadily increased in cells proceeding along S phase, with a maximal accumulation in cells in G2 phase (Figure 3a). Also, cyclins E and B showed the expected pattern, i.e. cyclin E was present exclusively in cells in late G1/early S phase (Figure 3b) and cyclin B in cells in G2/M (data not shown). After TPA treatment, cyclin E was expressed in both HEL and K562 cells, although the overall relative levels were higher in re-replicating HEL than in non re-replicating K562 cells.

During the first 12 h of treatment, it appears that cyclin A accumulates throughout the cell cycle in HEL cells, particularly in those with 4C DNA content, which remained positive up to 24 h. In contrast, in K562 cells the levels of the protein progressively went down, and almost no G2/M cells were positive 24 h after the treatment. In addition, the pattern of cyclin A expression in cells treated with TPA for 48 h showed dramatic differences in re-replicating and non-re-replicating HEL and K562 cells. Whereas in K562 cells cyclin A was nearly undetectable, not only in G1, but also in S and even G2/M phases (5% of cells), the protein was present in more than 42% of the HEL cells with DNA content 4C and >4C. Thus, with the exception of those cells that remained arrested in G1 phase (with DNA content = 2C), the basal level of expression was higher in all TPA-treated HEL cells. However, cells in distinct cell cycle phases showed different levels of the protein: cyclin A expression raised to G2 levels in those cells corresponding to the 4C and 8C peaks (G2/M re-replication cycle phases), falling in those with intermediate DNA content (S re-replication cycle phase). Interestingly though, such levels never went down below S phase levels, indicating that, although cyclin A actually cycles in the re-replicating cells, the succession of events appears to correspond to S-G2/M-S-G2/M, without cells having to proceed to an equivalent G1 phase.

The analysis of cyclin E expression also revealed significant differences between re-replicating and non-re-replicating cells (Figure 3b). Thus, although the levels of cyclin E were similar or slightly higher in exponentially growing K562 compared to HEL cells, the presence of the phorbol ester resulted in different effects in each cell line. Whereas in HEL cells the cyclin E levels steadily increased during the first 24 h, in K562 cells the proportion of cyclin E expressing cells went down in the same time-course, although some cells remained positive after 24 h independently of the cell cycle position. It looks, therefore, as if in HEL, and to some extent in K562 cells, the treatment with TPA results in a maintained expression of cyclin E throughout the cell cycle. Thus, the main difference between re-replicating HEL and non-re-replicating K562 cells is the accumulation of both cyclins E and A in G2/M phase cells in the first hours of the TPA treatment.

Altogether, these results strongly suggest that it is the continuous synthesis of cyclin A, due to cyclin E expressing G2 cells which allows megakaryoblastic cells to maintain an active DNA replication, although no cell division occurs.

Cyclin A can be detected in individual polyploid HEL cells and bone marrow megakaryocytes

In order to assess whether cyclin A is present in individual polyploid cells, we analysed by immunofluorescence HEL cells or treated with TPA for 96 h,

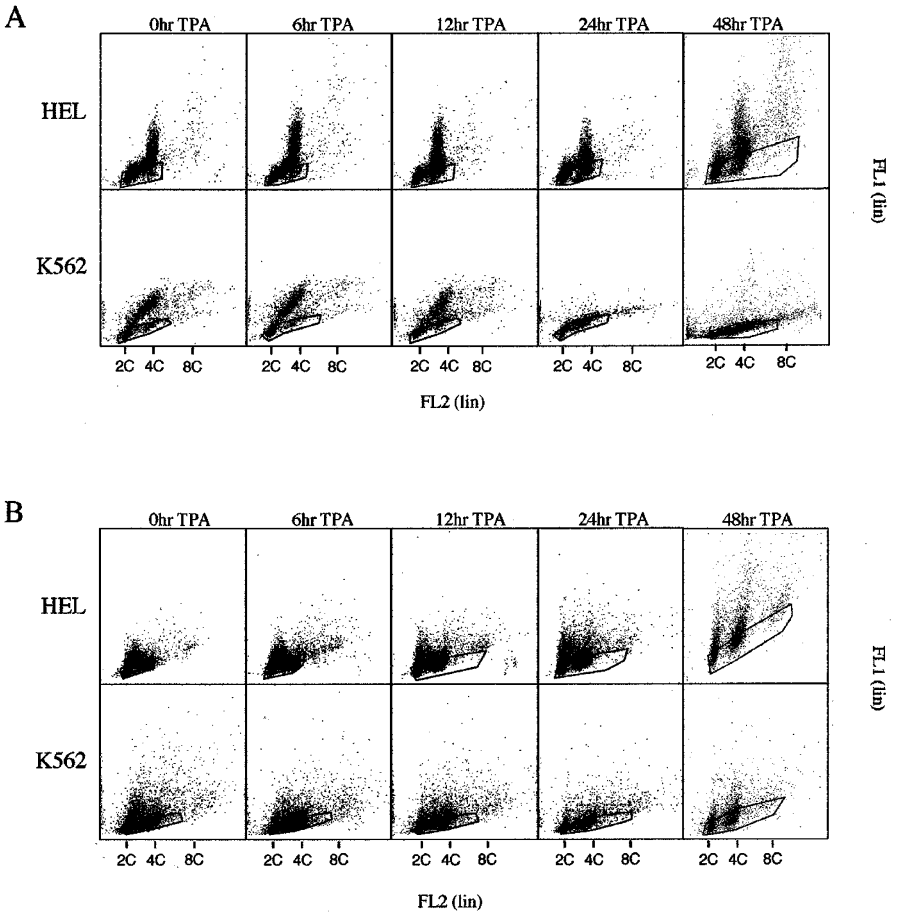

Figure 3 Changes in cyclin A and E expression within the time of TPA induction in re-replicating HEL and non re-replicating K562 cells. The diagrams show a flow cytometric analysis of HEL and K562 cells which were either growing exponentially or had been treated with TPA for 6, 12, 24 and 48 h. Expression of cyclin A was detected by direct immunofluorescence using a FITC-conjugated antibody (**a**) or anti-cyclin E/BODIPY-FITC-conjugated goat anti-mouse IgG (**b**), as described in Materials and methods (FL1, vertical axis, linear scale), and total DNA content was monitored by propidium iodide staining (FL2, horizontal axis, linear scale). The positions of cells stained in parallel with the isotype control are indicated by the overlayed polygon

after which time approximately 40% of the cells appear to have DNA content equal to or higher than 4C. Cells were labelled with FITC-conjugated anti-cyclin A antibodies and the presence of polylobulated nuclei was assessed by counterstaining nuclear DNA with propidium iodide. Observation of at least five different fields revealed that the percentage of cyclin A positive cells was approximately 40% in the exponentially growing cell preparation (data not shown). A similar percentage of positive cells was detected within the TPA-treated population, 80% of them corresponding to cells with polylobulated nuclei, indicating that HEL cells that have re-replicated their DNA do contain cyclin A. A representative field is shown in Figure 4a,b. The preparations analysed by confocal microscopy showed that cyclin A co-localizes with the labelled DNA, although a proportion of the protein was also detected in the cytoplasm (Figure 4c).

That the presence of cyclin A in polyploid megakaryocytes is not due to a peculiar feature of the leukaemic cell lines or due to the effect of the TPA treatment is indicated by the fact that mature megakaryocytes in bone marrow smears also contained cyclin A (Figure 4h). The megakaryocytic nature of giant, polyploid cells was assessed by staining with anti CD41 antibodies (Figure 4i). We can then conclude that polyploidization of both established megakaryoblastic cell lines and bone marrow megakaryocytic cells is associated with the presence of cyclin A.

Taken together, these results suggest that cyclin A is expressed during DNA re-replication of megakaryocytic cells, and therefore that its presence is compatible with the relief of the re-replication block.

Loss of cyclin A expression in non re-replicating K562 is due to inhibition of transcriptional activity in the presence of TPA

In order to investigate the link between the ability to enter a re-replication cycle and the maintenance of cyclin A protein levels in the presence of TPA, we analysed the ability of the cyclin A promoter to drive the expression of a luciferase reporter gene (luc) in the presence or absence of TPA in HEL and K562. Cells were transfected with the cycA[−875]luc construct which contained the luciferase gene under the control of sequences from −875 to +37 bp of the human cyclin A promoter. The transfected cells were split into two aliquots 5 h after transfection and one aliquot was treated with 10^{-8} M TPA. After 48 h, the cells were collected and the luciferase activity was assayed in whole cell extracts. As can be seen in Figure 5a, the cyclin A promoter appears to be repressed in non-re-replicating K562 cells when treated with TPA, whereas

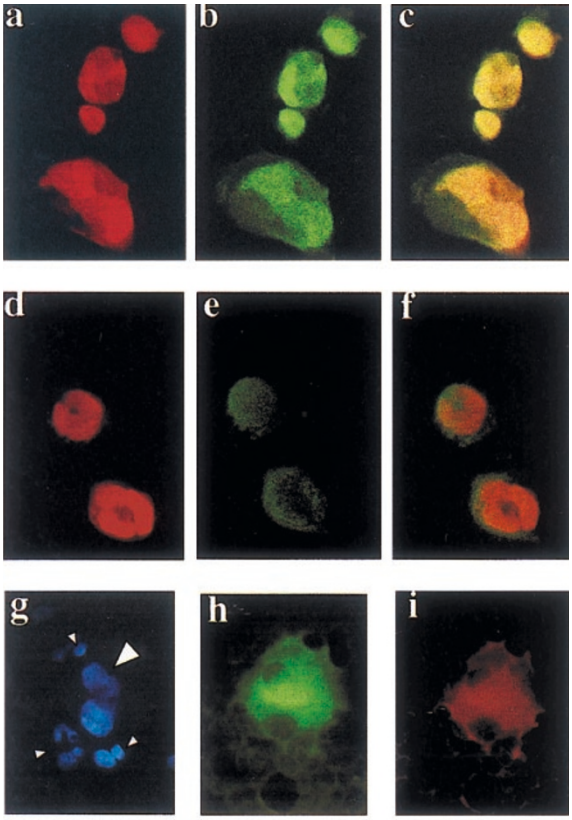

Figure 4 Cyclin A is present in re-replicating HEL cells and polyploid megakaryocytes. TPA-treated HEL cells (**a**–**f**) and bone marrow smears (**g**–**i**) were fixed in paraformaldehyde and stained with propidium iodide (**a**,**d**), DAPI (**g**), FITC conjugated anti-cyclin A antibody (**b**,**h**), FITC-conjugated goat anti-mouse IgE (**e**) or anti-CD41/TRITC-conjugated antimouse IgG (**i**). HEL cells preparations were analysed by confocal microscopy (**c**). Representative micrographs of these preparations are shown. (**a**–**f**) 1000× magnification; (**g**–**i**) 630× magnification. The arrows in (**g**) show the polylobulated megakaryocyte nucleus (big) and non-megakaryocytic nuclei (small)

in HEL cells full promoter activity is maintained. The maintenance of cyclin A promoter activity in the presence of TPA was also observed in another re-replicating megakaryoblastic cell line, MEG01, which was included in this experiment in order to check whether the results obtained in HEL cells were related to a particular feature of this cell line. In contrast, the megakaryocytic specific *gp*IIb gene promoter linked to the luciferase reporter was stimulated 4–10-fold in the presence of TPA in both K562 and HEL, and also in MEG01 cells, reflecting the expression of the *gp*IIb integrin which increases when both re-replicating and non-re-replicating megakaryoblasts are induced to differentiate (Figure 1a and data not shown).

It has been described that cyclin A transcription is directly regulated by cyclin E-cdk2 activity. In order to assess whether the TPA-driven inhibition of cyclin A promoter activity is due to an insufficient cyclin E-cdk2 activity, as suggested in Figure 5a, K562 were co-transfected with the cycA[−875]luc reporter construct together with the pcDNA3-cyclin E expression plasmid. The repression of cyclin A promoter activity observed in TPA-treated cells was reversed by the co-transfection of exogenous cyclin E in a dose-dependent manner; however, the expression of exogenous cyclin E in HEL cells had little or no effect in either the presence or absence of TPA (Figure 5b). This suggests that when cyclin E protein levels are above a certain threshold level then the inhibitory effect of TPA on the cyclin A promoter is overcome.

The direct involvement of an active cyclin E/cdk2 complex in the activation of the cyclin A promoter in TPA-treated cells was assessed by co-transfection of cycA[−875]luc and pcDNA3-cyclin E together with vectors expressing the cyclin E-cdk2 inhibitors $p21^{cip1}$ or $p27^{kip1}$. As a control we also tested the effect of co-expression of the cyclin D-cdk inhibitor p16. It can be seen from Figure 5c that the ability of cyclin E to overcome the TPA inhibitory effect on the cyclin A promoter could be specifically inhibited by $p21^{cip1}$ and $p27^{kip1}$, but was unaffected by p16.

Constitutive over-expression of cyclin E renders K562 cells permissive to enter a re-replication cycle in the presence of TPA

In order to determine whether the increase in cyclin E expression levels was responsible for the entrance into megakaryocytic re-replication cycles, we decided to overexpress the protein in non re-replicating K562 cells.

K562 cells were transfected with the expression vector pcDNA3 containing the human cyclin E cDNA (Hinds *et al*., 1992) under the control of the CMV promoter. We first analysed the effect in transient experiments, similar to those shown for cyclin A promoter transactivation experiments. As shown in Figure 6a, transient expression of cyclin E in K562 cells determined the appearance of polyploid cells after treatment with TPA.

In order to further characterize cyclin E effect, we decided to isolate stably transfected cells. Five G418-resistant clones were isolated and analysed for the expression of exogenous cyclin E. Since this cDNA encodes an alternatively spliced form of cyclin E which retains full biological activity, transgene expression can readily be distinguished from the major form of the endogenous protein (Resnitzky *et al*., 1994; Resnitzky and Reed, 1995). As can be seen in Figure 6b, two out of the five isolated clones (KEB and KEC), showed positive expression of the exogenous protein, which we calculated by densitometric analysis to be respectively 2–3 and 3–5 times that of the endogenous protein. The levels of protein expression also corresponded to the levels of RNA as determined by RT–PCR (data not shown). In parallel, K562 cells were transfected with an empty pcDNA3 vector and of the five isolated clones one, clone KCT, was chosen at random as a G418-resistant control.

Analysis of KCT, KEB and KEC clones showed that no apparent phenotypic changes could be detected in terms of cell morphology, as revealed by May-Grünwald-Giemsa staining, or the cell surface expression of megakaryocytic (CD61) or erythrocytic (glycophorin) antigens (data not shown). Both KEB and KEC cells showed a similar rate of cell proliferation compared to the parental cell line K562 and the G418-resistant cells KCT (data not shown). The only apparent difference that was detected was found in the proportion of cells undergoing S phase in cultures that had reached high density (data not shown), but not in exponentially growing cells (Figure 6d). From these analyses it appears that constitutive

expression of cyclin E does not affect the phenotype of K562 cells.

In order to study whether the constitutive expression of cyclin E would affect the behaviour of K562 cells in terms of establishment of a re-replication cycle, the clones KEB and KEC, as well as control KCT cells, were treated with 10^{-8} M TPA for a 48- or 96-h period. As can be seen in Figure 6d, treatment of both cyclin E over-expressing K562 clones with TPA resulted in a dramatic increase in cells with DNA content equal to or higher than 4C, the extent of this effect correlating with the amount of exogenously expressed cyclin E. The proportion of polyploid cells seen in TPA-treated KEC (57%) and KEB (50%) was similar to that obtained with HEL or MEG01 cells (Figure 6d, García and Calés, 1996). These data are summarized in Table 1. As it was observed for re-replicating HEL cells, both TPA-treated KEB and KEC extracts contained cyclin E-associated kinase activity (Figure 6c). Also, around 50% of KEB and KEC cells appeared to incorporate BrdU during a 8 h pulse labelling, in contrast with control KCT clone, of which only 10% of cells were BrdU stained (Figure 6e). Moreover, 70–75% of the KEB and KEC BrdU-positive population corre-

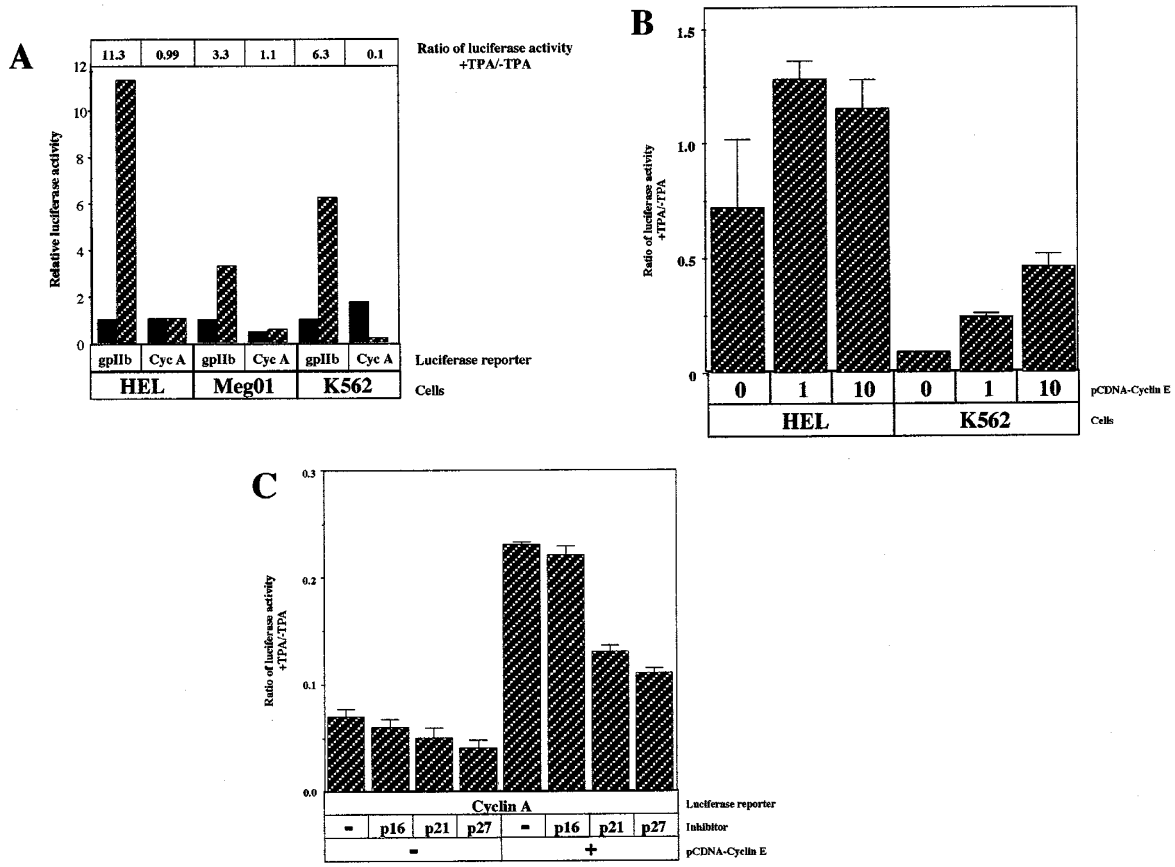

Figure 5 TPA down regulates cyclin A promoter activity in cells incapable of re-replication. (**a**) 2×10^6 of HEL, MEG01 and K562 cells were transfected by electroporation with 2 μg of either the *gp*IIb-luc or cycA[−875]luc reporter constructs. The cells were divided into two aliquots, to one of which was added 10^{-8} M TPA. Cells were collected and assayed for luciferase activity after 48 h. The filled and cross-hatched bars respectively represent untreated and TPA-treated cells. The activity of the *gp*IIb promoter has been arbitrarily set to a value of one for each of the three cell lines transfected and all other values are given relative to this activity. The fold stimulation of the promoter induced by TPA is shown at the top. (**b**) Transient expression of cyclin E overcomes the TPA-induced inhibition of the cyclin A promoter in K562 cells. HEL or K562 cells were transfected, treated with TPA and assayed for luciferase activity after 48 h. Each transfection included 2 μg of the cyclin A-luciferase reporter construct cycA[−875]luc and the indicated amount (μg) of pcDNA3-cyclin E expression plasmid. The total amount of DNA added was kept constant by the addition of the empty vector pcDNA3. The cyclin A promoter activities are presented as the ratio of luciferase activity in TPA-treated versus untreated cells. (**c**) Inhibitors specifically directed to cyclin E-cdk2 prevent the cyclin E mediated relief of TPA-induced inhibition of the cyclin A promoter. K562 cells were co-transfected with 2 μg of cyclin A-luciferase reporter and, where shown, 5 μg of pcDNA3-cyclin E expression plasmid as described in (**b**). In addition, 5 μg of plasmid expressing the indicated kinase inhibitors was included

Table 1 Cell cycle status of KCT, KEB and KEC cells untreated or treated with phorbol ester

	Percentage of cells								
	KCT			*KEB*			*KEC*		
Phase of cell cycle	*Control*	*+TPA/48 h*	*+TPA/96 h*	*Control*	*+TPA/48 h*	*+TPA/96 h*	*Control*	*+TPA/48 h*	*+TPA/96 h*
G1 (2C)	54	65	62	47	28	29	48	27	23
S (2C–4C)	17	10	10	30	13	14	28	15	20
G2 or re-replicating (>4C)	29	26	28	23	59	57	24	58	57

sponded to cells with a DNA content of 4C or higher, whereas positive KCT cells corresponded to cells in S phase (Table 2). Western blot analysis of cell cycle proteins revealed that the constitutive expression of cyclin E does not result in major changes in the levels of G2/M or G1/S regulators, compared with the parental cells (data not shown), except for cyclin A protein, the expression of which appeared to be re-established in TPA-treated KEB and KEC clones (see next section).

Together, these results show that constitutive expression of cyclin E determines the TPA-induced entry of K562 cells into a re-replication cycle.

Constitutive expression of cyclin E prevents TPA-induced repression of the cyclin A promoter and down-regulation of the protein in TPA-treated K562 cells

In order to investigate whether TPA treatment of cyclin E expressing clones resulted in the presence, as in HEL cells, or the absence, as in the parental K562 cells, of cyclin A expression, we performed transactivation experiments similar to those reported above.

KCT, KEB and KEC cells were transfected with the cycA[−875]luc reporter construct, and the luciferase activity was determined in parallel in cells treated or untreated with TPA. In contrast to the control clone

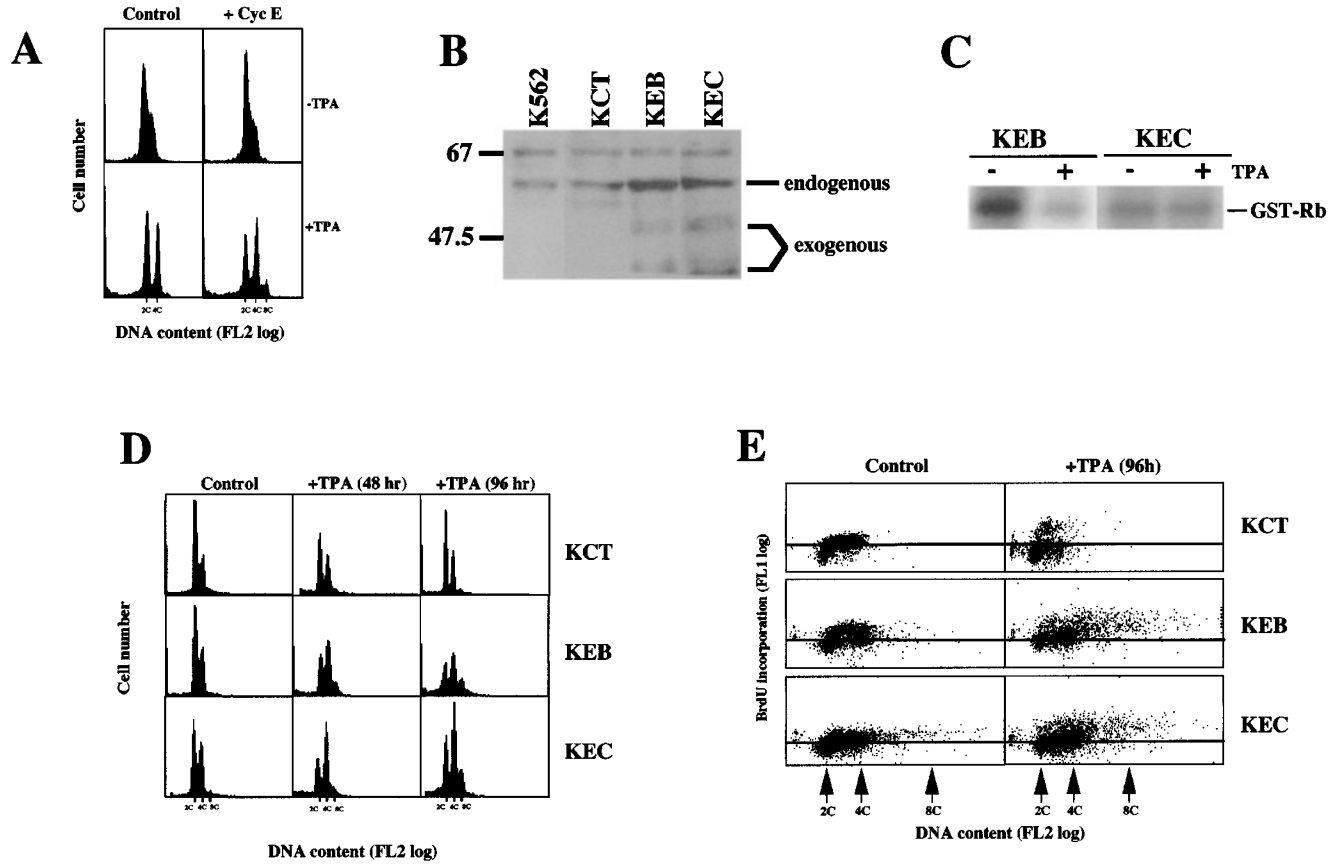

Figure 6 Over-expression of cyclin E restores associated kinase activity and enables TPA-induced polyploidy in K562 cells. (**a**) Transient expression of cyclin E. Cells (2×10^6) were transfected by electroporation with 10 μg of pCDNA3 or pCDNA-cyclin E. The cells were divided 24 h after transfection into two aliquots, to one of which was added 10^{-8} M TPA. Cells were collected and assayed for DNA content after a further 48 h by propidium iodide staining as described in Figure 1b. (**b**) Cyclin E protein levels. Total cell extracts were prepared from K562, the control KCT and cyclin E over-expressing clones KEB and KEC as described in Materials and methods. Protein (30 μg) was electrophoresed in SDS–PAGE and detected by Western blot with anti-cyclin E antibody. (**c**) Cyclin E-associated kinase activity was determined in KEB and KEC cells untreated (−) or treated with 10^{-8} M TPA for 48 h (+) as described in Figure 2. (**d**) KCT, KEB and KEC cells were cultured in the absence (control) or presence of 10^{-8} M TPA for 48 or 96 h. Cells were assayed for their DNA content by flow cytometry as described in Figure 1b. (**e**) Analysis of nuclear DNA content and the proportion of cells entering S phase. The diagram shows a flow cytometric analysis of cells grown in the absence or presence of TPA for 96 h. Cells that incorporated BUdR (included in the last 8 h of the incubation period) were detected by indirect immunofluorescence (FL1, vertical axis, logarithmic scale) and total DNA content was monitored by propidium iodide staining (FL2, horizontal axis, linear scale). The positions of peaks representing cells with a DNA content of 2, 4 and 8C are indicated

Table 2 Incorporation of BrdU into pulse-labelled cells

	Percentage of cells					
Cell cycle phase	*KCT*		*KEB*		*KEC*	
incorporating BrdU	*Control*	*+TPA/96 h*	*Control*	*+TPA/96 h*	*Control*	*+TPA/96 h*
Total	57	10	55	54	52	56
S phase	100	100	95	30	95	25
4C cells	0	0	5	70	5	75

KCT, which is indistinguishable from the parental K562 cells, the expression of luciferase was equal or even increased upon TPA-treatment of both cyclin E expressing cells KEB and KEC (Figure 7a). These

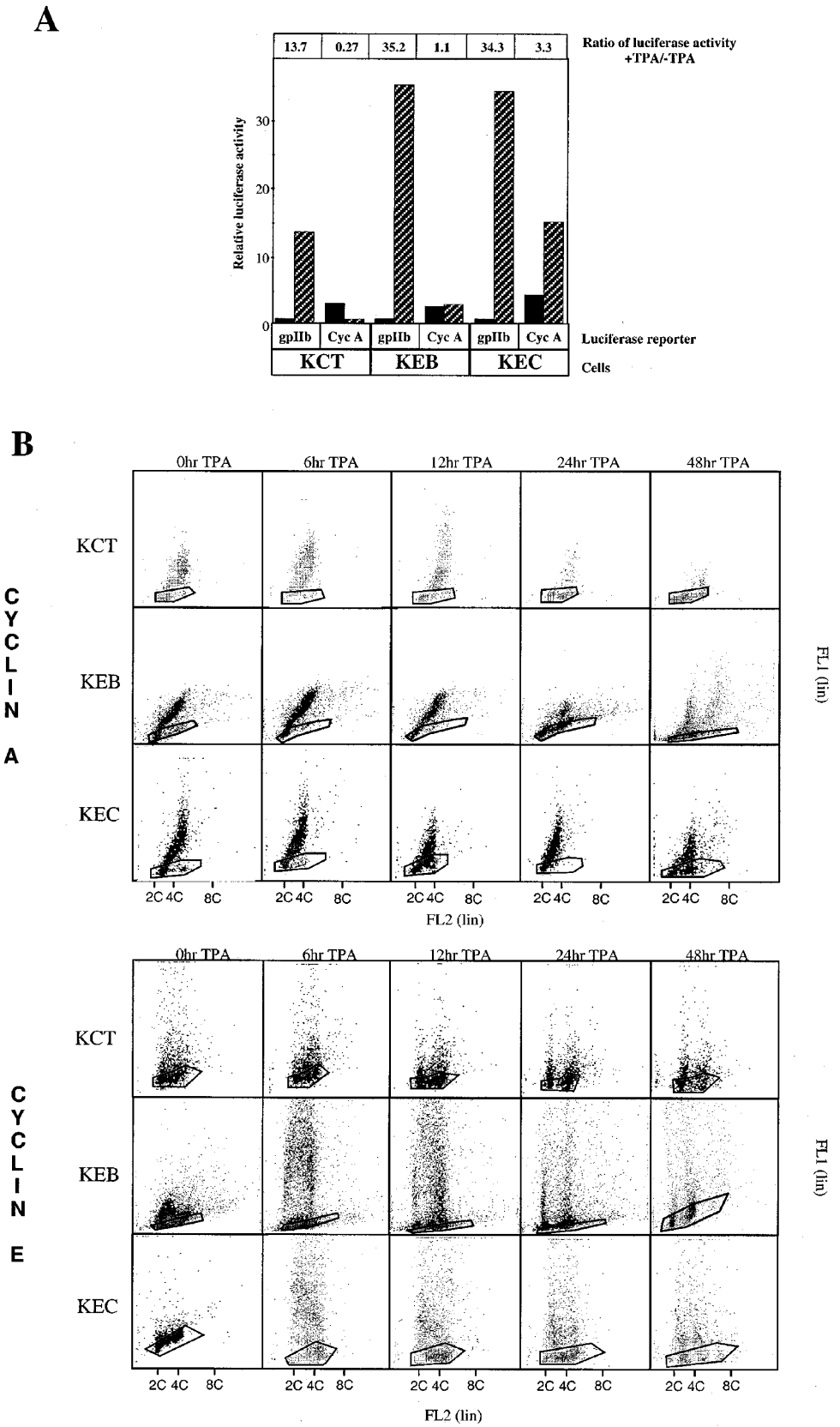

Figure 7 Cyclin E over-expression affects cyclin A expression in TPA-treated cyclin E over-expressing K562 derived cells. (**a**) TPA-induced inhibition of the cyclin A promoter and downregulation of the protein is prevented in KEB and KEC clones. Transfections were performed as described in Figure 5 using the control clone KCT or the cyclin E over-expressing clones KEB and KEC. (**b**) Re-replicating K562 derived clones accumulate cyclins E and A in the first 24 h and exhibit continued expression 48 h after TPA induction. The control KCT, KEB and KEC clones were treated with TPA for 6, 12, 24 and 48 h and stained simultaneously for cyclin expression and DNA content as described in Figure 3

results indicate that by constitutively expressing cyclin E, cyclin A promoter activity is no longer inhibited by TPA in K562 cells.

To determine the extent and timing of cyclin A expression, cytometric analyses were then performed at 0, 6, 12, 24 and 48 h after TPA treatment. As can be seen in Figure 7b, at the time of the treatment (exponentially growing cells), all three clones follow the expected pattern of cyclin A expression. However, during the first 12 h of treatment, it appears that cyclin A accumulates throughout the cell cycle in both KEB and KEC cells, particularly in those with 4C DNA content, which remained positive up to 24 h. In contrast, in KCT, as in K562 cells, the levels of the protein progressively went down, and almost no G2/M cells were positive 24 h after the treatment.

It appears, therefore, that in re-replicating KEB and KEC cells, the pattern of cyclin A expression is equivalent to that seen in HEL cells, thus further strengthening the idea that cyclin E determines the maintenance of cyclin A expression, allowing the entrance into a re-replication cycle.

The analysis of cyclin E in KEB and KEC cells surprisingly revealed that even though a significant proportion of the protein is expressed from a constitutive promoter, its levels follow the regular pattern of expression in exponentially growing cells: the highest expressing cells being in G1/S phase. After being treated with TPA, the protein accumulated at levels nearly ten times higher than in exponentially growing cells in all the cell cycle phases. Interestingly though, after 24 h, high levels of the protein were found in cells with 4C DNA content, indicating that although the expression is maintained throughout the cell cycle, the TPA-treated cells that have reached G2/M phase accumulated the protein. Thus, as it occurred for HEL and K562 cells, the main difference between re-replicating KEB and KEC cells and non-re-replicating KCT cells is the accumulation of both cyclins E and A in G2/M phase cells in the first hours of the TPA treatment.

Altogether, these results strongly suggest that it is the continuous synthesis of cyclin A due to cyclin E expression in G2/M cells which allows megakaryoblastic cells to establish an active re-replication.

Discussion

One of the hallmarks of megakaryocytic lineage differentiation is the formation of a very large, polyploid cell. Although the biological significance of polyploidization is not yet fully understood, circumstancial evidence in human thrombocytopoenia (Kristensen *et al*., 1988; Hegyi *et al*., 1991) and studies in animals (Kuter and Rosenberg, 1990) suggest that the process by which megakaryocytes increase their nuclear DNA must be regulated in order to efficiently maintain blood platelet counts. In this paper, we have examined the role of the factors that govern the G1/S transition in the establishment of the peculiar cell cycle that results in the re-replication of nuclear DNA without an intervening complete mitosis in megakaryoblastic cells.

To date, little is known about the molecular mechanisms by which this cell type is able to overcome the tight control that determines the proper succession of S and M phases in the majority of somatic cells. In fact, it has only been demonstrated recently that megakaryocytes in fact enter mitosis. Thus, two independent laboratories have shown that differentiated megakaryocytes proceed to early mitotic phases, which include nuclear membrane breakdown and sister chromatid limited segregation (Vitrat *et al*., 1998; Nagata *et al*., 1997). In one of these studies, it is even shown that cyclin B1 undergoes proper proteolysis (Vitrat *et al*., 1998). We have also observed that in re-replicating HEL cells, cyclin B1 cycles as it does in exponentially growing cells. Hence it is clear that some mitotic events take place, and this leads to two fundamental questions: (i) how do megakaryocytes 'leave' mitosis?; and (ii) how do they subsequently enter a new S phase? It could be that endomitotic cells, after progressing through a normal G2 phase (i.e., down-regulating G1/S cyclins E and A), skip part of the late mitotic events, then proceed to a G1 phase in order to rebuild all of the G1/S machinery. However, some of the studies performed in megakaryoblastic cell lines have clearly shown that re-replication can occur in the absence of a totally active mitotic complex. For instance, re-replication takes place in megakaryoblastic cells lacking cyclin B1 (Zhang *et al*., 1996), cdc2 (Datta *et al*., 1996) or cdc25C, even though cyclin B and cdc2 are present (García and Calés, 1996; this paper). Also, some early data showed that treatment of megakaryoblastic cells with agents that provoke mitotic arrest results in the entrance into re-replication cycles (van der Loo *et al*., 1993; García and Calés, 1996, unpublished observation). All this evidence points at an alternative hypothesis, that is, that megakaryoblastic cells are prepared to re-enter S phase after exiting an incomplete mitosis, or even directly from G2 phase. It is therefore of great interest to elucidate the mechanisms by which megakaryocytes enter such extra S phases. We have addressed this question in this paper.

By using an experimental system in which we can compare two cell lines which respond similarly to the same stimulus by differentiating towards mature megakaryocytic forms, and yet show substantial differences in terms of establishing a re-replication cycle, we have been able to assess an important role of cyclin E. Recently, both cyclin D3 and cyclin E have been shown to be actively complexed with cdk2 during polyploidization of HEL cells (Datta *et al*., 1998). Previously, we had ourselves already determined that in megakaryoblastic HEL and MEG01 cells cyclin E levels were apparently maintained after TPA treatment (García and Calés, 1996). Here, we have extended these data, showing that cyclin E is able to confer the ability to establish a re-replication cycle on differentiating K562 cells which do not otherwise become polyploid after TPA treatment. These data are in accordance not only with the primary role of this cyclin, that is, the entrance into S phase (Dulic *et al*., 1992; Resnitzky *et al*., 1994), but also with its essential role in other re-replicating systems such as *Drosophila* embryonic cells (Duronio *et al*., 1996; Knoblich *et al*., 1994), maize endosperm (Grafi and Larkins, 1995), and mouse trophoblasts (MacAuley *et al*., 1998). Interestingly, our data also suggest that cyclin E is present in differentiating cells undergoing re-replication cycles, in contrast with what occurs along a mitotic cycle of exponentially growing cells. We cannot determine whether this is due to

continous synthesis or to lack of programmed proteolysis, or both, as suggested by metabolic labelling experiments which show that cyclin E is actively synthesized in HEL cells at short (3-h) or long (96-h) term after TPA treatment (García, Frampton and Calés, 1998, unpublished results).

Our results also suggest that cyclin A expression correlates with the megakaryoblastic cells ability to establish a re-replication cycle. In fact, we show that mature megakaryocytes express cyclin A, as has been reported by others (Zhang *et al*., 1996). This is compatible with the fact that megakaryocytes proceed through a complete G2 phase before entering mitosis. It is also expected that to undergo S phase, cells have to express cyclin A, as it has been established for proliferating cells (Ohtsubo *et al*., 1995; Zindy *et al*., 1992; Girard *et al*., 1991). Surprisingly, though, in other re-replicating systems including mouse trophoblasts (MacAuley *et al*., 1998; Sprenger *et al*., 1997), cyclin A appears not to be needed for extra S phases. This apparent discrepancy could be based upon fundamental differences between the biological significance of the respective re-replication cycles. In *Drosophila* and trophoblast cells, endocycles are intended to produce polytenic chromosomes (Varmuza *et al*., 1988; Smith and Orr-Weaver, 1991) and it has even been shown that in *Drosophila* endocycles only early S phase takes place, and late replication is skipped (Lilly and Spradling, 1996). In contrast, the polyploidization of megakaryocytes results in the multiplication and segregation of normally duplicated chromosomes. Complete S and G2 phases should then be maintained, and this could explain the need for cyclin A expression, the role of which in the progression of S into G2 phase has been suggested not only in mammalian cells (Pagano *et al*., 1992; Pines, 1993), but also in the *Drosophila* embryo and developing eye (Dong *et al*., 1997; Sprenger *et al*., 1997).

Our results suggest a direct involvement of cyclin E in maintaining cyclin A expression at the transcriptional level. Although this role for cyclin E has already been shown in fibroblasts (Zerfass-Thome *et al*., 1997), here we show that this also occurs in haemopoietic cells. In non re-replicating K562 cells, TPA appears to inhibit cyclin A transcription resulting in cell cycle arrest, this inhibition being relieved by constitutive expression of cyclin E. The effect of phorbol esters in terms of down-regulating cyclin A also occurs in non-megakaryocytic haemopoietic cell lines; for example in TPA-treated progranulocytic HL-60 (Horiguchi *et al*., 1993) or promonocytic U937 cells (García and Calés, 1996). However, in these latter cells most of the G1/S (including cyclin E) and G2/M proteins appear to be down-regulated. What we have found to be dramatically different between exponentially growing and re-replicating cells is the presence of cyclin E in cells that have trespassed S phase. We propose that it is this very circumstance in which a G2/M cell (with down-regulated cyclins A and B) already has cyclin E, which would then allow the entrance into and maintenance of a re-replication cycle by driving cyclin A expression. In fact, transient expression of cyclin A alone also determined the entrance of K562 cells into re-replication cycles (García, Frampton and Calés, 1998, unpublished results), although not as efficiently as cyclin E alone, and co-transfection of both did not potentiate such effect. In other re-replicating systems, e.g. *Drosophila* and mouse trophoblasts, in which both cyclins A and B are no longer expressed, cyclin E appears to cycle as it does in mitotic cycles (Follette *et al*., 1998; Sauer *et al*., 1995). Moreover, continuous expression inhibits re-replication in *Drosophila* embryos (Follette *et al*., 1998; Weiss *et al*., 1998). Our results show that at least the establishment of an extra cycle can occur in the presence of cyclin E and that in fact the accumulation of cyclin E in cells in late S and G2 phases appears to directly relate to the ability to establish a re-replication cycle. This could be interpreted as not compatible with models that suggest the need of reducing cdk activity in order to license a cell to initiate a new round of DNA replication (Jallepalli and Kelly, 1997). Our results show that during megakaryocytic endomitosis, both cyclins A and B levels fluctuate as in a mitotic cycle, which most likely results in a reduction of cdk1 activity. This cdk1 variations could be sufficient to allow the cell to re-initiate DNA replication, without the need to progress through G1 phase, given that cyclin E could drive pre-replication events.

A great deal of work is being performed in order to unravel the regulatory mechanisms that determine that DNA is replicated once and only once per cell cycle. We believe that studying megakaryocytic endomitosis will provide a very useful tool on how this control can be overcome.

Materials and methods

Cell culture

Cells were cultured in RPMI medium supplemented with 10% (v/v) foetal calf serum (Gibco), 2 mM L-glutamine and 60 mg/ml gentamicin. Cells were maintained at 37°C under 5% CO_2/95% air in a humidified incubator. In all experiments, exponentially growing cells at $0.15-0.20\times10^6$ cells per ml were subcultured into Nunc 96, 24 or 6-wells plates with or without appropriate treatment. The number of viable cells was determined by Trypan Blue exclusion in a haemocytometer chamber. To induce megakaryocytic differentiation, cells were grown in the presence or absence of 10^{-8} M o-tetradecanoylphorbol 13-acetate (TPA, Sigma) for 48–96 h

Constructs used in stable and transient transfections

The cyclin E expression vector pcDNA3-cyclin E was generated by ligating a 1.6 kbp *Hind*III fragment (−57 to +1542) from pADN-HU4 cycE into the *Hind*III site of the expression vector pcDNA3 polylinker (InVitrogen). Similarly, the $p27^{kip1}$ expression plasmid was constructed from an *Eco*R1 fragment derived from pBluescript-hp27FL containing the full length $p27^{kip1}$ cDNA (Polyak *et al*., 1994) ligated into the *Eco*RI site of pcDNA3. Cyclin A and the inhibitors $p21^{cip1}$ and p16 were expressed from CMV promoter driven vectors.

The reporter plasmid cycA[−875]luc containing the human cyclin A promoter was derived from the pXP1 promoterless luciferase vector (deWet *et al*., 1987) into which was cloned a PCR amplified fragment of the human cyclin A gene from residues −875 to +37 (Henglein *et al*., 1994). The 5′ and 3′ oligonucleotides used to PCR amplify this region were 5′-AGGCACGTATAGTTAAGAGAGT-3′ and 5′-AGCCAAAGACGCCCAGAGAT-3′. *gp*IIb-luc was also constructed from a PCR generated fragment of the murine *gp*IIb gene promoter cloned into pXP1; sequences from −538 to +45 (Denarier *et al*., 1993) were amplified using the

oligonucleotides 5′-CTGGTTGAGGCCGCCCAAAG-3′ and 5′-AAGCTCTGGCCATCTTCCTTCT-3′.

Transient transfection assays

Megakaryoblastic cell lines were transfected by electroporation. 2×10^6 cells were transfected with 2.5 μg of the luciferase reporter and variable amounts of the expression plasmids by electroporation at 125 μF/300 V in a 0.4 cm cuvette. Electroporated cells were then divided into two aliquots and plated in normal growth medium. After a period of 12 h to allow recovery of the cells, one aliquot was treated with TPA as described above. Forty-eight hours after electroporation the cells were recovered and cell extracts for assaying of luciferase activity were made by three cycles of freeze-thaw lysis. The transfection efficiency was detected by 2.5 μg of pGFPN1 under the same conditions resulting on 25% of cells transfected.

Isolation of stable clones

K562 cells were transfected by electroporation. 5×10^6 cells were transfected with 5 μg of linearized pcDNA3 or pcDNA3-cyclin E plasmids. After 24 h, cells were washed with phosphate-buffered saline and resuspended in fresh medium supplemented with 500 μg/ml G418 antibiotic (Gibco). Cells were subcultured in 96-well plates at 1×10^5 cells per ml. Single-cell clones were isolated by limiting dilution of the G418-resistant cells.

Cell surface characterization of cells

Surface immunofluorescent staining of cells was performed by incubating cells with the appropriate dilution of first antibody at 4°C for 10 min followed by washing and incubation with fluorescein (FITC) conjugated goat anti-mouse second antibody at 4°C for 20 min. Stained cells were analysed on a Becton Dickinson FACScan flow cytometer using CellQuest software.

In situ *subcellular localization of cyclin A expression by fluorescence microscopy*

HEL cells undergoing exponential growth or treated with TPA for 96 h were plated on glass coverslips treated with 1% of gelatin (Sigma). The cells were fixed in 3.7% paraformaldehyde in PBS for 15 min at room temperature. After quenching in 50 mM ammonium chloride for 5 min, the cells were permeabilized with 0.2% Triton X-100 in PBS for 8 min, blocked with 5% serum in PBS for 20 min and incubated with FITC conjugated anti-cyclin A antibody or the corresponding IgE control (Pharmingen) for 30 min at room temperature. After treatment for 30 min at room temperature with RNAse (preheated at 95° for 15 min) nuclei were stained for DNA by incubation with 10 mg/ml propidium iodide. The preparations were mounted on glass slides in Mowiol and were examined in a confocal microscope.

Bone marrow smears were fixed and labelled as described for HEL cells except that prior to incubation with the anti-cyclin A or control antibodies, the preparations were incubated sequentially with anti-CD41 antibody (AP-2, Pidard *et al*., 1983) for 30 min at room temperature and TRITC-conjugated goat anti-mouse IgG1. Nuclei were visualized by DAPI staining.

Flow cytometric characterization of DNA content

To determine DNA content, cells were harvested by centrifugation at 500 *g* for 5 min, washed in ice-cold phosphate-buffered saline and then resuspended in 50 mM sodium citrate, 50 μg/ml propidium iodide, 0.1% Nonidet P-40. Cell cycle analysis was performed using a FACScan analyser using CellQuest software (Becton Dickinson). All histograms represent 10 000 cells.

To measure the proportion of cells undergoing S phase, cells were labelled for a defined time with 20 μM BUdR (Boehringer Mannheim) and then pelleted and fixed in 70% ethanol for 15 min at room temperature. The genomic DNA was denatured by incubation of the fixed cells in 0.5 ml 1N HCl/0.5% Tween 20 at 37°C for 15 min. After washing twice in 1 ml ice cold PBS/1% BSA the cell pellet was resuspended in 50 μl anti-BUdR monoclonal antibody (Amersham) diluted 1 : 50 in PBS/0.1% Triton X-100. After 20 min on ice followed by one wash in PBS/1% BSA, bound anti-BUdR was detected by incubating the cells in 50 μl 1 : 40 diluted goat anti-mouse Ig-FITC for 30 min on ice. The cells were washed and then analysed by flow cytometry in the presence of propidium iodide as described above.

Immunoblotting

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; 1 μM each phenylmethylsulfonyl fluoride (PMSF), sodium fluoride, β-glycerophosphate, sodium vanadate and EGTA; 5 μg/ml each Na-p-tosyl-L-lysine chloromethyl ketone (TLCK), leupeptin and pepstatin; and 5 mM sodium pyrophosphate. Thirty μg of protein extracts were subjected to SDS–PAGE (7% polyacrylamide gels for detection of pRB and cyclin A, 12% polyacrylamide gels for detection of cdc2 and cyclin B and 10% polyacrylamide gels for detection of cdc25C, cyclin E, and cdk2). The gels were transferred to Immobilon P membranes (Millipore) for 1 h at 2 mA/cm^2 on a semi-dry transfer apparatus (LKB). Filters were blocked for 2 h at room temperature in Tris-buffered saline containing 0.1% Tween 20 (TTBS), and 5% skimmed dry milk, and incubated overnight at 4°C with the appropriate primary antibody diluted in TTBS. Working dilutions were: 1/5000 for anti-cyclin A/B1; 1/1000 for anti-cyclin E, cdc2, cdk2 and cdc25C; and 1/250 for anti-pRB. The filters were washed three times for 5 min in TTBS and then incubated for 1 h at room temperature with goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase (Nordic Immunological Laboratories). After extensive washing in TTBS, signals were detected using the enhanced chemi-luminiscence (ECL, Amersham) system.

Immunocytochemical detection of cyclins

For the simultaneous analysis of cell cycle and cyclin expression, cells were washed in PBS and fixed in suspension in 10 ml cold 75% ethanol. After 30 min at 4°C, the fixed cells were pelleted, washed once in PBS/0.1% NaN_3/10% FCS (wash buffer) and then resuspended in 5 ml cold 0.25% Triton X-100 in wash buffer and incubated at 4°C for 5 min. Permeabilized cells were pelleted after addition of 10 ml wash buffer. One hundred μl aliquots of cells in wash buffer were incubated for 30 min at room temperature with either 20 μl FITC-conjugated anti-cyclin A or B1 or isotype control antibody (IgE or IgG1 respectively), or with 1 μg anti-cyclin E and an appropriate irrelevant IgG1 control. All specific and isotype control antibodies were obtained from Pharmingen. Anti-cyclin E binding was detected after washing by a 30 min incubation in 100 μl of a 1 : 200 dilution of goat anti-mouse Ig BODIPY-FITC (Molecular Probes) in wash buffer containing 10% goat serum. All labelled cells were washed again and resuspended in 5 μg/ml propidium iodide in PBS. The analysis was performed using a FACScan analyser using CellQuest software (Becton Dickinson).

Immunoprecipitation and kinase activity determination

Extracts from TPA-treated or untreated cells were isolated as described for Western blot analysis. Three hundred micro-

grams of total protein were precleared by dilution in 1 ml of lysis buffer and incubation with 50 μl of 50% v/v protein A-Sepharose (Sigma) precoated with normal rabbit serum. Tubes were gently rocked for 2 h at 4°C. Samples were centrifuged, and the resultant supernatant was split into two aliquots and incubated for 2 h at 4°C in the presence of agarose-conjugated anti-cyclin E (Santa Cruz) or an equivalent amount of agarose-conjugated normal rabbit IgG. For cdk2-associated kinase assays, anti-cdk2 polyclonal antibody (Pharmingen) followed by protein A-Sepharose isolation was performed. Immunoprecipitates were washed four times with 1 ml of ice-cold lysis buffer and once with 1 ml of kinase buffer (50 mM Tris-HCl, pH 7.4; 10 mM magnesium chloride; 1 mM dithiothreitol). Pellets were then resuspended in 40 μl of kinase buffer with 250 ng of the carboxy-terminal part of retinoblastoma protein (Large Pocket-GST fusion: pRb aminoacids 379–928, kindly provided by Dr Manuel Serrano). Reactions were initiated by the addition of 5 μM ATP and 10 μCi of γ-[^{32}P]ATP (3000 Ci/mmol) and incubated at 30°C for 30 min. Phosphorylated GST-Rb was further purified by incubation of the resultant supernatant with GSH-agarose (Pharmacia). 4× Laemmli sample buffer was then added and samples boiled for 5 min. Twenty-five microliters of each reaction was analysed by SDS–PAGE on a 10% polyacrylamide gel, and bands were detected by autoradiography.

Acknowledgments
We are grateful to Dr Manuel Serrano for crucial suggestions and reagents (p16 expression vector and GST-Rb recombinant protein). We thank Dr Jonathon Pines for polyclonal antibodies against cyclins A and B1, and for the cyclin A expression vector; Dr Steven Reed for cyclin E cDNA; Dr Chris Norbury for cdc2 antibodies; Dr Steven Elledge for p21 cDNA; Dr Joan Massagué for p27 cDNA; and Dr David Mason for anti-glycophorin A (JC159a) and anti-CD61 (Y2/51) monoclonal antibodies. We also thank Anthony Crawford and Carmen Domínguez for technical assistance and Miguel Vidal for critical reading of the manuscript. This work was supported by grants from the Fundación para la Investigación y Formación en Oncología to C Calés, a Wellcome Trust Senior Biomedical Fellowship to J Frampton, a grant from the Association for International Cancer Research to J Frampton, and a joint collaborative grant from the British Council-Ministerio de Educación y Cultura to C Calés and J Frampton. P García was a recipient of a short-term EMBO fellowship.

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