The Cell Cycle in Polyploid Megakaryocytes Is Associated with Reduced Activity of Cyclin B1-dependent Cdc2 Kinase*

(Received for publication, June 13, 1995, and in revised form, November 29, 1995)

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The platelet precursor, the megakaryocyte, matures to a polyploid cell as a result of DNA replication in the absence of mitosis (endomitosis). The factors controlling endomitosis are accessible to analysis in our megakaryocytic cell line, MegT, generated by targeted expression of temperature-sensitive simian virus 40 large T antigen to megakaryocytes of transgenic mice. We aimed to define whether endomitosis consists of a continuous phase of DNA synthesis (S) or of S phases interrupted by gaps. Analysis of the cell cycle in MegT cells revealed that, upon inactivation of large T antigen, the cells shifted from a mitotic cell cycle to an endomitotic cell cycle consisting of S/Gap phases. The level of the G1/S cyclin, cyclin A, as well as of the G1 phase cyclin, cyclin D3, were elevated at the onset of DNA synthesis, either in MegT cells undergoing a mitotic cell cycle or during endomitosis. In contrast, the level of the mitotic cyclin, cyclin B1, cycled in cells displaying a mitotic cell cycle while not detectable during endomitosis. Comparable levels of the mitotic cyclin protein, Cdc2, were detected during the mitotic cell cycle or during endomitosis; however, cyclin B1-dependent Cdc2 kinase activity was largely abolished in the polyploid cells. Fibroblasts immortalized with the same heat-labile oncogene do not display reduced levels of cyclin B1 upon shifting to high temperature nor do they become polyploid, indicating that reduced levels of cyclin B1 is a property of megakaryocytes and not of the T-antigen mutant. We conclude that cellular programming during endoreplication in megakaryocytes is associated with reduced levels of cyclin B1.

The development of hematopoietic cells consists of few stages: the commitment of pluripotent stem cells to differentiate rather than to remain in the resting G0 phase or to proliferate, lineage restriction and maturation of these committed cells, and synthesis of cell-specific gene products. Although the mechanisms by which cells withdraw from the stem cell pool are presently unknown, it was suggested that the initial steps may be stochastic (Suda et al., 1983). Thereafter, growth factors support progenitor cells to develop along particular differentiation pathways (Metcalf, 1989). In the megakaryocytic lineage, endomitosis involving DNA synthesis in the absence of mitosis, as well as platelet production, are stimulated by thrombopoietin, recently isolated (Kaushansky et al., 1994; Wendling et al., 1994; Kuter et al., 1994; de Sauvage et al., 1994; Chang et al., 1995). The regulation of the cell cycle and of endomitosis in this cell type has not been explored yet.

The major events common to all eukaryotic cell cycles are the replication of chromosomes during S phase and their segregation during mitosis. The dependences of S phase and mitosis on each other ensure orderly progression through the cell cycle (Hartwell and Weinert, 1989). However, in some developmental situations, chromosome replication and segregation can be uncoupled. For example, a G2/S cycle takes place without any intervening mitosis in cells during the early development of the Drosophila embryo (Smith and Orr-Weaver, 1991), as may also be the case in ploidizing megakaryocytes. However, in most cells, the dependence of S phase upon mitosis (M) and of M on S phase are strictly observed. The regulation points of the cell cycle are the G2/S and G2/M transitions, for which the kinase activity of Cdk2 or Cdc2, respectively, is crucial. These kinases are controlled through association with regulatory subunits known as cyclins (Nasmyth, 1990; Evans et al., 1983; Westendorf et al., 1990, Pines and Hunter, 1990). The G2/M transition is dependent on the activity of the Cdc2 kinase, activation of which requires association of Cdc2 with B-type cyclins and dephosphorylation on tyrosine (Draetta et al., 1989; King et al., 1994). Regulators of the G2/S transition include the cyclin B1-dependent Cdc2 kinase (or its homologue Cdc28 in S. cerevisiae) associated to cyclin A (Pines and Hunter, 1990; Bartlett and Nurse, 1990). In dams, these two different cyclins, A and B, show a similar periodicity in their synthesis and degradation, but in mammalian cells the levels of cyclin A rise near the beginning of S phase and the levels of cyclin B peak at the entry to mitosis (Evans et al., 1983; Westendorf et al., 1990; Pines and Hunter, 1990). The regulation of the G1 phase was studied first in S. cerevisiae, where it was found that cyclins, cln1, cln2, and cln3 (CLN genes), control progression through G1, by modulating the activity of Cdc28 kinase (Hadwiger et al., 1989; Wittenberg et al., 1990). Subsequently, equivalents to these Cln cyclins have been identified, cyclins D1, D2, and D3 (reviewed by Reed, 1991).

Past studies of megakaryocyte development, endomitosis, and maturation have been hampered because of the rarity of megakaryocytes in bone marrow and because of the lack of a pure megakaryocytic cell line that can enter and complete a normal maturation cycle. Different leukemia cell lines derived from hematopoietic progenitors have been used in studying the biochemistry as well as gene regulation of the megakaryocytic lineage. Some human erythroleukemic cell lines exhibit myeloid, erythroid, as well as megakaryocytic markers (Martin and Papayannopoulou, 1982; Tablío et al., 1983; Ravid et al., 1993a), while other cell lines (Greenberg et al., 1988; Sledge et al., 1986; Adachi et al., 1991) are enriched with megakaryocytic markers, but require exposure to a substance such as phorbol 12-myristate 13-acetate in order to ploidize. This later agent, being pluripotent, may induce changes in cyclin expression.
unrelated to megakaryocyte ploidy, as demonstrated on a non-megakaryocytic cell line HL60 (Akizyme et al., 1993). We have recently generated a megakaryocytic cell line, MegT, by targeted expression of the temperature-sensitive form of large T antigen in megakaryocytes of transgenic mice, via the platelet factor four tissue-specific promoter (Ravid et al., 1993b). MegT cells which become polyploid upon inactivation of the oncogene were used to determine the role of different cyclins in promoting endomitosis. Our data suggest that once large T antigen is degraded, the cells undergo endomitosis while containing low levels of cyclin B1 and low activity of the mitotic kinase.

MATERIALS AND METHODS

Culture Conditions—MegT cells (clone 37C1) were grown in a liquid culture, all as described before (Ravid et al., 1993b). To induce ploidy, 1 x 10^6 cells were seeded into a 75-cm² culture flask and incubated in 5% CO₂ at 39.5 °C for 4–5 days (Ravid et al., 1993b). Cells were counted by hemocytometer, and cell viability was followed by staining with Trypan Blue. For synchronization experiments, cells were cultured for 24 h at the indicated temperature after which the cells were shifted to a medium containing 0.1% horse serum. Forty eight hours later, fresh medium containing 20% horse serum was added to the cells. The attached cells as well as the detached cells were collected separately at different times and were subjected to various analyses. Immortalized mouse proerythroblasts transformed by heat-labile (tsA58)T antigen (generous gift of Dr. Harvey Ozer) were cultured as described elsewhere (Hubbard-Smith et al., 1992).

Immunoprecipitation and Western Blotting—Immunoprecipitation and Western blot analyses were performed as described before (Xiong et al., 1992). To this end, MegT cells adhering to the culture dish and nonadhering cells were collected separately by trypsinization or by spinning down cells (380 x g, 5 min) in the medium, respectively. Cells were washed twice with cold phosphate-buffered saline (PBS) (136 mM NaCl, 8 mM Na₂HPO₄, 2.6 mM KCl, 1.4 mM KH₂PO₄, pH 7.4) by centrifugation and lysed in lysis buffer (0.5% Nonidet P-40, 50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 1% sodium phenylmethylsulfonyl fluoride, 10 μg/ml N-tosyl-L-phenylalanine chloromethyl ketone, 10 μg/ml soya trypsin inhibitor) followed by centrifugation at 15,000 x g for 5 min. Lysates, each containing 100 μg of protein in 400 μl of lysis buffer, were preclarified by incubation with normal mouse IgG and 40 μl of Zosarin (fixed and killed Staphylococcus aureus protein A, Zymed Laboratories, Inc., San Francisco, CA) for 1 h at 4 °C followed by centrifugation at 15,000 x g for 5 min. Antibody was added to the clarified lysate and incubated for 1 h at 4 °C. Protein A-Sepharose CL-4B (Pharmacia Biotech Inc.) was added at a volume of 40 μl and incubated at 4 °C for an additional hour. Immunoprecipitates were washed three times with lysis buffer, resuspended in sodium dodecyl sulfate (SDS) sample buffer, and separated on 12% SDS-polyacrylamide gel (SDS-PAGE) (Laemmli and Favre, 1973). Protein assays were done as recommended by the manufacturer (Bio-Rad Laboratories).

For Western blotting, 10 μg of lysed proteins were separated on 7.5% or 12% SDS-PAGE (Laemmli and Favre, 1973) and electrophoretically transferred from the gel onto an Immobilon-P membrane (Millipore) in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. The transfer was done as recommended by the manufacturer (Bio-Rad Laboratories). Polyacrylamide gel electrophoresis; MPF, mitosis promoting factor; kb, kilobase(s).

RESULTS

Expression of Messages for Different Cyclins in MegT Cells—Proliferating MegT cells were synchronized by serum deprivation, and RNA was prepared from cells harvested at different time points of the cell cycle. The RNA was subjected to Northern blot analysis using different cyclin cDNAs as probes. As shown in Fig. 1, MegT cells expressed cyclins A, B1, D1, and D3 mRNAs, but to a lesser extent B2 mRNA, and not D2 mRNA. It should be pointed out that the B2 and D2 cDNAs used as probes detected the corresponding mRNA from mouse fetal bone marrow, confirming that the probes used were capable of recognizing the murine messages (not shown). Indeed, as described for other systems, B-type and D-type cyclins are differentially expressed in different tissues (Chapman and Wolgemuth, 1992, 1993). Correlation between the Level of Large T Antigen and of the Mitotic Cyclin in Diploid and Polyploid MegT Cells—We noted that once MegT cells were shifted to the temperature which is not permissive for stability of large T antigen, a fraction of the

1 The abbreviations used are: PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; MPF, mitosis promoting factor; kb, kilobase(s).
cells remained adhering to the dish while the other fraction detached from the dish. Then, nonadhering cells, representing 34.19% (n = 6) of the cells at 4 days postculturing, appeared as round and larger cells. At the permissive temperature (34°C), all cells remained adhering to the culture dish (Fig. 2). In the current study, we performed separate ploidy analyses on the detached and adhering cells cultured at the same nonpermissive temperature as compared to MegT cells cultured at 34°C. Under the later conditions, all cells were adhering to the dish and consisted of 2N (diploid) and 4N cells (Fig. 3). At the nonpermissive temperature, the fraction of cells attached to the plate also consisted of 2N and 4N cells (Fig. 3B). In contrast, the majority of the detached cells were 4N and 8N cells (Fig. 3C). In a previous study, we found that the megakaryocyte-promoting factor, thrombopoietin (Kuter et al., 1994), did not have a significant effect on the ploidy state of MegT cells.2 Recently, these cells were also analyzed for their ability to express c-mpl, the receptor for thrombopoietin (Wendling et al., 1994). We found that the level of expression of c-mpl in MegT cells was low, revealed only by the polymerase chain reaction, but not by Northern blot analysis (not shown). This indicated that signaling pathways critical for reaching a ploidy state higher than 8–16N are not active in these cells.

Of most interest was the observation that the level of large T antigen, as revealed by Western blot analysis, was notably reduced in the cells in suspension (polyploid cells) but not in the adhering cells (mostly diploid cells) cultured at the same nonpermissive temperature (Fig. 4A). We then determined the level of components of the mitosis promoting factor (MPF), being cyclin B and Cdc2, in both fractions of cells. As shown in Fig. 4A, the level of cyclin B1 protein, but not of Cdc2, was significantly reduced in the polyploid cells. Northern blot analysis indicated, however, that the level of cyclin B1 mRNA was not changed significantly in polyploid cells (Fig. 4B). In order to establish whether a reduced level of cyclin B is a property of a T-antigen mutant cell line or rather a property of polyploid megakaryocytes, we determined the level of T antigen and of cyclin B in immortal fibroblasts (AR5) transformed by origin-defective SV40 encoding a heat-labile T antigen (Hubbard-Smith et al., 1992). At high temperature, these cells display a reduced growth rate, but no hyperploidy (Resnick-Silverman et al., 1991). Western blot analyses (Fig. 5) indicated that, although T antigen was degraded in AR5 cells incubated at high temperature, the level of cyclin B was not altered.

The Activity of the Mitotic Kinase during the Cell Cycle in Synchronized MegT Cells—Pulse labeling with radiolabeled thymidine was used to determine the profile of DNA synthesis in MegT cells, synchronized by serum deprivation and cultured

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2 K. Ravid, D. Kuter, and R. Rosenberg, unpublished data.
at the nonpermissive temperature. Upon release from synchronization, at successive time points, the adhering and nonadhering cells were labeled for 1 h with [3H]thymidine and collected separately. During 40 h in culture, two cycles of DNA synthesis were observed in MegT cells adhering to the dish, each spanning about 18 h. The value of [3H]thymidine incorporation at the first peak of DNA synthesis was lower than the peak value during the second cycle, and the cell number doubled at the end of the second cycle, all as expected during a mitotic cell cycle (Fig. 6A). In contrast, each cycle of DNA synthesis in the detached cells was completed within 10 h with a short gap between the S phases (Fig. 6B). The cell number remained constant at the end of both cycles, as expected during endoreduplication. The second peak of DNA synthesis reached a value for [3H]thymidine incorporation lower than the one expected in case all cells would have undergone endoreduplication. It should be pointed out, however, that also in the case of primary bone marrow megakaryocytes only a fraction of the cells continues endomitosis for several cycles to reach a high ploidy state (Rovolic, 1974). Immunoprecipitation of equal amounts of proteins prepared from the mitotic and endoreduplicating MegT cells revealed that the activity of the mitosis promoting factor (MPF), consisting of cyclin B-dependent Cdc2 kinase, was high during mitosis in the replicating MegT cells while hardly detectable in the polyploid cells (inset in Fig. 6).

The Level of Different Cyclins in Synchronized MegT
Cells—We also sought to determine the level of G₁ phase, G₁/S, and M phase cyclins at the onset and offset of DNA synthesis in synchronized MegT cells cultured at the nonpermissive temperature. As shown in Fig. 7A, Western blot analyses revealed that during the mitotic cell cycle the level of the G₁/S cyclin, cyclin A, was slightly higher at the onset of DNA synthesis while elevation of cyclin B1 level started at S phase, as expected before entry to mitosis. During the endomitotic cell cycle (Fig. 7B), the levels of cyclin A and of the protein Cdc2 were significantly high, but cyclin B1 was barely detectable. As to the large T antigen, it was readily detectable during the mitotic cell cycle, but, undetectable in the polyploid cells. These results further suggested that turning off the oncogene was a prerequisite for shifting from a mitotic cell cycle to endomitosis. It should be also pointed out that, during the mitotic cell cycle, the level of cyclin A cycled moderately, while it did not seem to cycle during the endomitotic cell cycle. It is possible that we were unable to detect a rapid transient decrease in cyclin A during the short G phase of the endomitotic cell cycle. The level of the G₁ phase protein, cyclin D3, for which a high level of mRNA was detected (Fig. 1) was also determined. Cyclin D3 level was high at time points corresponding to the G₂ phase (Fig. 6) during the mitotic cell cycle while moderate changes were observed in the level of this cyclin during endomitosis (Fig. 8).

DISCUSSION

Endomitosis, involving DNA replication in the absence of mitosis, can occur in three types of cells: those in which the endoreplicated chromosomes are not synapsed or visible, those in which cyclic condensation of the chromosomes is observed, and, in some cases, those in which multinucleate cells have been referred to as polyploid also. In the case of the megakaryocytic lineage, the endoreplicated DNA is all concentrated in one nucleus (Metcalf, 1989). However, it is not certain yet if polyploid megakaryocytes enter only prophase involving chromosome condensation or prophase as well as metaphase, involving chromosome condensation and spindle formation but skip anaphase, or whether the cells skip all stages in the G₂ and M phases to directly enter a G₁ phase. While electron microscopic analyses of different stages of mitosis in megakaryocytes are underway, we sought to investigate the cyclin composition of megakaryocytes undergoing endomitosis.

In a previous study, we generated several clones of megakaryocytic cell lines by targeted expression of the temperature-sensitive form of large T antigen in transgenic mice, via the tissue-specific platelet factor four (PF4) promoter (Ravid et al., 1993b). These cell lines express several megakaryocytic markers, such as the glycoprotein GP11b, and acetylcholine esterase, and at the permissive temperature adhered to the dish and contain high levels of large T antigen (Ravid et al., 1993b). In the current study, we have chosen to investigate a clone which allowed us to perform analyses of the mitotic and endomitotic cell cycle at the same nonpermissive temperature. When MegT cells (clone 37C1) were cultured at the nonpermissive temperature, only a fraction of the cells (about 30%) had undetectable levels of large T antigen and solely those cells appeared as round cells in suspension with high ploidy nuclei. Because of the leaky nature of this conditional oncogene, large T antigen may not have been completely destroyed in the rest of the cells which remained adhering to the dish. Nevertheless, this feature was taken as an experimental advantage, since it allowed us to compare levels of different cyclins in diploid and polyploid megakaryocytes cultured at the same elevated temperature. Our results indicated that cells expressing high levels of large T antigen were unable to initiate endoreduplication.

In a recently published study, additional transgenic mice carrying the temperature-sensitive large T antigen under the control of the PF4 promoter have been generated (Robinson et al., 1994). Upon aging, some of these mice developed megakaryocytic leukemias displaying aberrations in megakaryocyte morphology and low platelet counts. Transgenic mice in which megakaryocytes reached high ploidy level seemed to express minute amounts of the oncogene (Robinson et al., 1994). We aimed to define whether the megakaryocytic cell cycle during endomitosis consists of a continuous S phase or of Gap/S phases and to determine which cyclins are involved in this process. Pulse-labeling of MegT cells with [³H]thyidine revealed that during endomitosis DNA synthesis was not continuous, but, rather, interrupted by a short gap. The level of the G₁ phase cyclin, cyclin D3, cycled during the mitotic cell cycle in MegT cells and rose at the gap phase during endomitosis. This latter result further suggested the existence of a G₁ phase during endomitosis in megakaryocytes. During this process of endoreduplication, the whole cell cycle was quite short, span-
ning about 10 h, in correlation with a previous study in rat primary bone marrow megakaryocytes (Odell et al., 1968). Further cyclin analyses in MegT cells revealed no significant differences in the level of cyclin A and in the level of Cdc2 protein during a mitotic or endomitotic cell cycle. However, while the cyclin B1 level rose at the onset of mitosis in continuously doubling MegT cells, it was hardly detectable in the polyploid cells. In accordance, the activity of cyclin B1-dependent kinase was low. Interestingly, the reduced level of cyclin B1 could not be attributed to low level transcription, as the level of cyclin B1 mRNA was not reduced in polyploid cells. The mechanism of cyclin degradation, a highly selective process, is not well understood. The amino-terminal sequences have been shown to play a critical role in targeting cyclins to the ubiquitin degradation pathway (Hershko et al., 1991), the activation of which occurs at the onset of anaphase. It is plausible then that cyclin B1 is either not stable or not translated in polyploid cells.

Although two types of cyclin B were described in mammalian cells, B1 and B2 (Chapman and Wolgemuth, 1993), it is not clear yet which of these cyclins plays a role in different stages of mitosis in eukaryotic cells and if they are able to substitute for each other. While the level of cyclin B1 protein was reduced during endomitosis in MegT cells, we were unable to determine the level of cyclin B2 protein because of the lack of an antibody that recognizes mouse cyclin B2. It should be pointed out, however, that cyclin B1 mRNA is the predominant one in MegT cells (Fig. 1). Nevertheless, we do not exclude the possibility that small amounts of B2 mRNA may lead to a significant amount of B2 protein in MegT cells. In many systems tested, the lack of cyclin B1 alone is sufficient to drive endoreduplication. Endoreduplication in some Drosophila cell types is indeed associated with a lack of cyclin B1 (Lehner and O’Farrell, 1990). Also, the metaphase II arrest in mouse oocytes is controlled through destruction of cyclin B1 (Kubiak et al., 1993).

Initiation of endoreduplication in different systems depends on the availability of Cdc2 kinase and cyclin B, both composing an active M phase promoting factor (MPF). Thus, certain treatments, such as inhibitors of protein kinases in mammalian cells or high levels of the protein encoded by rum1, which inhibits the mitotic kinase in fission yeast, block M phase and induce repeats of S phase (Usui et al., 1991; Moreno and Nurse, 1994). In the filamentous fungus Aspergillus nidulans, the NIMA protein kinase is required in addition to MPF for the M phase (O’Connell et al., 1994). Overexpression of this kinase in different systems, including human cells, resulted in chromatin condensation without other aspects of mitosis (O’Connell et al., 1994). Although functional homologues of NIMA in mammalian cells have not been described yet, some kinases have been reported to have homology to the catalytic domain of NIMA.

**Fig. 7. The levels of different cyclins in synchronized MegT cells.** Cell lysates were prepared separately from adhering (A) mitotic and detached (B) endomitotic MegT cells, cultured at 39.5°C, at different hours postrelease from synchronization. The cell lysates were subjected to Western blot analysis, using 12% acrylamide gel. Equal loading of protein prepared from endomitotic cells in suspension (C) and adhering mitotic (D) cells (10 μg of protein/lane) was confirmed by brief staining (1–2 min) of the blots with 0.1% Ponceau S in 5% acetic acid followed by destaining in water for 2 min and photography, followed by a 10-min rinse in water prior to reaction with the indicated antibodies. Lane M contains a ladder of molecular mass markers. Large T antigen, cyclin A, cyclin B1, and Cdc2 appeared with the molecular masses of 82, 60, 55, and 34 kDa, respectively, on the blot.
**Fig. 8. Cyclin D3 levels in MegT cells.** Cell lysates were prepared separately from adhering (A) mitotic and detached (B) endomitotic MegT cells, cultured at 39.5 °C, at different hours postrelease from synchronization. The cell lysates were subjected to Western blot analysis, using 10% acrylamide gel. Equal loading of protein prepared from endomitotic cells in suspension (C) and adhering mitotic (D) cells (10 μg of protein/ lane) was confirmed by brief staining (1–2 min) of the blots for 2 min and photography, followed by a 10-min rinse in water prior to reaction with cyclin D3 antibody. Cyclin D3 appeared with a molecular mass of 33 kDa on the blot.

(Schultz and Nigg, 1993). These studies suggest that not all steps of mitosis are regulated by the MPF kinase. The fission yeast Schizosaccharomyces cerevisiae, temperature sensitivity cut8-563 mutation causes chromosome overcondensation and short spindle formation in the absence of cytokinesis, thus leading to the identification of a gene (cek1) encoding a novel protein kinase which complements the mutation that blocks anaphase (Samejima and Yanagida, 1994). The model proposed by O'Connell et al. (1994) involves condensation of chromatin by a NIMA homologue while other mitotic events down to anaphase are regulated by MPF. Anaphase is regulated by inactivation of MPF and may be regulated also by other kinases such as cdc15 analogues. If so, our model of polyol cyclin D3 cells with significantly reduced levels of cyclin B1 may be used for studying the roles of different mitosis-related kinases in endomitosis.

Acknowledgments—We thank Tony Hunter and Emma Lees for valuable insight, Dimitry Kamen for helpful technical assistance, and Harvey Ozer for the generous gift of immortal SVtsA/HF-A cells.

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J. Biol. Chem. 1996, 271:4266-4272.
doi: 10.1074/jbc.271.8.4266

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