Ubiquitin-dependent Degradation of Cyclin B Is Accelerated in Polyploid Megakaryocytes*

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During the endomitotic cell cycle of megakaryocytic cell lines, the levels of cyclin B1 and the activity of cyclin B1-dependent Cdc2 kinase, although detectable, are reduced as compared with megakaryocytes undergoing a mitotic cell cycle. The levels of cyclin A, however, are comparable during both cell cycles. The expression of cyclin B1 mRNA is also equivalent in proliferating and polyploidizing cells. In the current study, we found that the rate of cyclin B1 protein degradation is enhanced in polyploidizing megakaryocytes. This finding has led us to further investigate whether the ubiquitin-proteosome pathway responsible for cyclin B degradation is accelerated in these cells. Our data indicate that polyploidizing megakaryocytic cell lines and primary bone marrow cells treated with the megakaryocyte proliferation- and ploidy-promoting factor, the c-Mpl ligand, display increased activities of the ubiquitin-proteosome pathway, which degrades cyclin B, as compared with proliferating megakaryocytic cell lines or diploid bone marrow cells, respectively. This degradation has all the hallmarks of a ubiquitin pathway, including the dependence on ATP, the appearance of high molecular weight conjugated forms of cyclin B, and inhibition of the proteolytic process by a mutated form of the ubiquitin-conjugating enzyme Ubc4. Our studies also indicate that the ability to degrade cyclin A is equivalent in both the mitotic and endomitotic cell cycles. The increased potential of polyploid megakaryocytes to degrade cyclin B may be part of the cellular programming that leads to aborted mitosis.

During the mitotic cell cycle, cyclin B steadily accumulates at interphase and is rapidly degraded before the cells exit mitosis (reviewed in Ref. 1). Cyclin B degradation is essential for progression through to the next cell cycle. The specificity of cyclin B degradation is determined by the 9-amino acid motif conserved among the N termini of B-type cyclins (cyclin Box) (reviewed in Refs. 1 and 2). Mutation of the conserved arginine residue among the N termini of B-type cyclins (cyclin Box) served as a signal for ubiquitination (3, 4). Cyclin A and B2, but not B1, require binding to β34Cd2 for their proteolysis (5, 6). While some components involved in cyclin B ubiquitination and degrada-

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Polyploidy and Ubiquitination and Degradation of Cyclin B

MATERIALS AND METHODS

Culture Conditions—MegT cells (clone 3TC1) were grown in a liquid culture, as described before (19). To induce ploidy, 1 x 10^6 cells were seeded into a 75-cm² culture flask and incubated in 5% CO₂ at 37 °C for 5 days. The cells were counted by hemocytometer, and cell viability was followed by staining with trypan blue. The attached cells as well as the detached cells were separately collected and subjected to various analyses. A clone (Y10/L8057) of the mouse megakaryocytic cell line L8057 (20), selected by limited dilution based on its ability to express acetylcholinesterase (a unique marker for rodent megakaryocytes (21)) and to respond to 25 ng/ml PEG-rHuMGDF (generous gift of Amgen, Inc. Thousands Oaks, CA) by polyploidizing. Acetylcholinesterase activity was assayed as described before (9). Culture conditions for this cell line were as described elsewhere (20). Rat bone marrow cells were isolated from femurs also as described before (22). Cells were cultured in 5% CO₂ at 37 °C in a liquid culture under conditions that were previously shown to support maturation and ploidy of primary megakaryocytes (22, 23). The cells were cultured in the presence of Iscove’s modification of Dulbecco’s medium (IMDM) and 20% horse serum supplemented with penicillin (2000 units/ml), streptomycin (200 µg/ml), t-glutamine (0.592 mg/ml), and when indicated with 50 ng/ml PEG-rHuMGDF for 3 days prior to collection of large mature megakaryocytes.

Purification of Megakaryocytes—Polymerizing megakaryocytes of varying sizes (20-µm diameter and higher) were isolated from rat bone marrow cells derived from rat femurs (22) and cultured for 3 days in the presence of 50 ng/ml of PEG-rHuMGDF, to promote megakaryopoiesis, and in the presence of a mixture of growth factors including 1 unit/ml erythropoietin (Amen, 10 units/ml of granulocyte-macrophage colony-stimulating factor, and 5 units/ml of stem cell factor (Genzyme Corp., Boston, MA) to promote proliferation and survival of different bone marrow lineages, as we described before (22). The isolation procedure involved mesh filtration (nylon mesh screens from Spectrum Medical, Inc., Los Angeles, CA). To this end, clumps were initially removed by filtration through a 200-µm filter followed by filtration with a 17-µm filter, which allowed diploid cells to pass through the mesh. Cells retained on the filter were retrieved by washing the inverted filter with phosphate-buffered saline (136 mM NaCl, 8 mM NaH₂PO₄, 2.6 mM KCl, 1.4 mM KH₂PO₄, pH 7.4). This procedure was repeated twice. We confirmed by counting cells under the light microscope and by staining for the unique marker for megakaryocytes, acetylcholinesterase (21), that megakaryocytes were enriched to account for 60–70% of bone marrow cells (see Fig. 8A), a level comparable with the purification method involving elutriation (24). Because of the size and content of megakaryocytes, in comparison with the small diameter bone marrow cells, the enrichment of megakaryocytic material (e.g. protein) would be substantially higher.

Western Blotting—Y10/L8057 cells grown in suspension were collected by centrifugation (380 x g, 5 min) in growth medium, and MegT cells adhering to the culture dish were stripped from the plate with a cell scraper, while nonadhering cells were collected separately by centrifugation. Cells were washed twice with cold phosphate-buffered saline (10 mM Tris (pH 7.6), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). Cells were resuspended in 2 volumes of Spectromatic buffer (1 mM NaCl) and blocked for 1 h with TBST (Tris-buffered saline with 0.1% Tween 20) containing the following components in a final volume of 25 µl: 12 µM Tris (pH 7.5), 60 mM KCl, 3.5 mM MgCl₂, 1 mM ATP (or ATP-S), 20 mM creatine phosphate, 50–200 ng of recombinant cyclin B1 or cyclin A (Pharmingen), purified from salt by buffer exchange with an Amicon 30-kDa filtration unit (Amicon Beverly, MA). The reaction was carried out at 30 °C for the indicated time. When indicated, the cellular extract was pretreated with 0.15 units of apyrase (Sigma) for 10 °C or 30 °C, after which the reaction was processed in the absence of ATP or creatine phosphate. UbC4 and UbC2 were obtained as described elsewhere (28). Point mutations in UbC4 and UbC2 were generated by oligonucleotide-directed mutagenesis as described in Ref. 28.

Ploidy Analysis—Bone marrow was collected from femurs of rats as described before (22, 23). Marrows were suspended in 0.5 ml of CATCH buffer and incubated for 60 min at 4 °C with 4A5 monoclonal antibody ascites (generous gift of Sam Burstein, University of Oklahoma Health Sciences Center). The cells were then incubated with fluorescein-conjugated goat anti-rat IgG (Fab)₂ (BioSource International, Camarillo, CA) for 30 min at 4 °C. Staining with propidium iodide was followed by determination of DNA content of 4A5 positive cells using a FACScan flow cytometer (Becton-Dickinson) as we described previously (10). Y10/L8057 cells were stained with propidium iodide and subjected to ploidy analysis also as described before (10).

RESULTS

Levels of Cyclin B1 and Cdc2 and the Half-life of Cyclin B1 in the Proliferating and Polyploidizing Megakaryocytic Cells Y10/L8057—In the current study, we assayed for cyclin B1 and Cdc2 levels during polyploidization of a megakaryocytic cell line, Y10/L8057, which we subcloned from L8057 cells (20) (see “Materials and Methods”), based on its ability to polyploidize in response to the megakaryocyte ploidy-promoting factor PEG-HuMGDF (Fig. 1). In the presence of PEG-HuMGDF or of phorbol 12-myristate 13-acetate (PMA), a moderate or large fraction, respectively, of the cells underwent polyploidization, while the rest remained in the 2N proliferative mode (Fig. 1). Western blot analyses indicated that during polyploidization induced by these agents, the levels of cyclin B1, but not of Cdc2, were reduced (Fig. 2). We next explored the possibility that reduced levels of cyclin B reflected an accelerated degradation of this protein in polyploidizing cells. As shown in Fig. 3, the half-life of cyclin B1 in polyploid Y10/L8057/L8057 cells was substantially shorter than in proliferating cells. The enhanced degradation and reduced levels of cyclin B1 in polyploidizing megakaryocytes were not a result of cell cycle arrest, since cells were treated against the N-terminal domain of cyclin B between amino acids 1 and 21 (Pharmingen, San Diego, CA). This antibody cross-reacts with the corresponding murine protein, as tested by the manufacturers and by ourselves (10). When indicated, a polyclonal antibody to cyclin B1, which does not block exclusively the N-terminal region of cyclin B, was used (20). Limited amounts of this antibody were available to us, and thus most of the studies were performed with the commercial monoclonal antibody to cyclin B1. The blot was washed four times, each time for 10 min, and incubated for 1 h with appropriate horseradish peroxidase-labeled secondary antibody (Amersham Corp.) at 1:1500 dilution in TBST. The blot was washed four times (each for 10 min) with TBST, and the Enhanced Chemiluminescence system (Amersham) was used for detection of proteins, as instructed by the manufacturer.

Preparation of Cytoplasmic Extract and Degradation Assay—Cells were washed twice with phosphate-buffered saline by centrifugation (380 x g, 5 min). The volume of the pelleted cells was estimated, and the cells were washed again by centrifugation in the presence of 5 volumes of hypotonic buffer (10 mM Tris (pH 7.6), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). Cells were resuspended in 2 volumes of Spectromatic buffer and incubated on ice for 10 min prior to breaking by strokes in a glass homogenizer. Cells were spun down for 10 min in a microcentrifuge. Protein assay was performed on the supernatant as described in Ref. 10. Since adequate degradation was obtained with this preparation (27), we did not need to further clean the cytoplasmic extract by high speed centrifugation (100,000 x g). Degradation of recombinant cyclin B was monitored as follows. Cytoplasmic extracts (25 µg per ml PEG-rHuMGDF) were added to a reaction mixture containing the following components in a final volume of 25 µl: 12 µM Tris (pH 7.5), 60 mM KCl, 3.5 mM MgCl₂, 1 mM ATP (or ATP-S), 20 mM creatine phosphate, 50–200 ng of recombinant cyclin B1 or cyclin A (Pharmingen), purified from salt by buffer exchange with an Amicon 30-kDa filtration unit (Amicon Beverly, MA). The reaction was carried out at 30 °C for the indicated time. When indicated, the cellular extract was pretreated with 0.15 units of apyrase (Sigma) for 10 °C or 30 °C, after which the reaction was processed in the absence of ATP or creatine phosphate. UbC4 and UbC2 were obtained as described elsewhere (28). Point mutations in UbC4 and UbC2 were generated by oligonucleotide-directed mutagenesis as described in Ref. 28.

The abbreviations used are: PEG-HuMGDF, pegylated recombinant human megakaryocyte growth and development factor; IMDM, Iscove’s modified Dulbecco’s medium; ATP-S, adenosine 5'-O-(thiotriphosphate); PMA, phorbol 12-myristate 13-acetate; APC, anaphase-promoting complex; FCS, fetal calf serum.

1 The abbreviations used are: PEG-HuMGDF, pegylated recombinant human megakaryocyte growth and development factor; IMDM, Iscove’s modified Dulbecco’s medium; ATP-S, adenosine 5'-O-(thiotriphosphate); PMA, phorbol 12-myristate 13-acetate; APC, anaphase-promoting complex; FCS, fetal calf serum.
induced to polyploidize displayed high rates of DNA synthesis (Table I).

**Accelerated Degradation of Recombinant Cyclin B by a Ubiquitin-Proteosome Extract Derived from Polyploidizing Y10/L8057 Cells**—A shortened half-life of cyclin B could result from an enhanced activity of the ubiquitin-proteosome pathway in polyploidizing cells. To test this possibility, cytosolic extracts were prepared from proliferating megakaryocytes or cells undergoing endomitosis. These extracts were incubated with recombinant cyclin B1, and cyclin B1 degradation was followed. As shown in Fig. 4, a significant degradation of recombinant cyclin B was displayed by extracts from highly polyploidizing Y10/L8057 cells (treated with PMA), less from cells displaying moderate polyploidization (treated with PEG-rHuMGDF), but not in proliferating cells. A high level of cyclin B1 degradation (in PMA-treated cells) was dependent on the addition of ATP to the reaction, as typical of a ubiquitin-dependent degradation (30) (Fig. 4).

**Degradation of Cyclin A Is Not Accelerated in Polyploidizing Y10/L8057 Cells**—Cyclin A protein is highly expressed in the...
megakaryocytic cell line, MegT, in HEL cells (10, 31) and in Y10/L8057 cells undergoing polyploidization (Fig. 5A). Since cyclin A is also subjected to degradation via the ubiquitin pathway (reviewed in Ref. 32), we examined whether the ability of polyploid megakaryocytes to degrade this cyclin is accelerated as was the case for cyclin B1. Interestingly, and in accordance with the detection of high levels of cyclin A in polyploid megakaryocytes, the ability of this latter cell type to degrade recombinant cyclin A was comparable with the rate displayed by proliferating cells (Fig. 5B).

**Accelerated Degradation of Recombinant Cyclin B by a Ubiquitin-Proteosome Extract Derived from Polyploidizing MegT Cells**—MegT cells are megakaryocytes that have been immortalized with the temperature-sensitive large T antigen (19). At the permissive temperature (34 °C), the cells proliferate. At the nonpermissive temperature (39.5 °C), a fraction of the cells remain adhering to the dish and undergo a mitotic cell cycle, while the other fraction (in which T antigen is destroyed) detaches from the plate and undergoes an endomitotic cell cycle (10). During this endomitotic process, the level of cyclin B protein, but not of cyclin A protein nor of cyclin B mRNA, is reduced, as compared with proliferating cells (10). Cytosolic extracts were prepared from these proliferating megakaryocytes or from cells undergoing endomitosis. These extracts were incubated with recombinant cyclin B1, and cyclin B1 degradation was followed. As shown in Fig. 6A, a significant degradation of recombinant cyclin B was displayed by extracts prepared from polyploidizing cells. Cyclin B degradation was prevented in the presence of apyrase, which degrades ATP, or in the presence of the nonhydrolyzable (by the proteosomes) ATP analog, ATPγS. An additional hallmark of a ubiquitin-dependent degradation is the appearance of high molecular weight conjugates of cyclin B and ubiquitin. To detect these high molecular weight complexes, we also used a polyclonal antibody to cyclin B protein. This antibody, in contrast to the monoclonal antibody used in Fig. 6A, is not directed against the N-terminal domain of cyclin B and thus should recognize ubiquitin-bound cyclin B. Indeed, as shown in Fig. 6B, high molecular weight conjugates of recombinant cyclin B appeared only in polyploid cells, depending on the availability of ATP.

**PEG-rHuMGDF-treated Primary Bone Marrow Cells Display an Increased Ability to Degrade Recombinant Cyclin B**—To test that the phenotype observed in Y10/L8057 and MegT cells is not exclusive to these cell lines, we prepared extracts from primary rat bone marrow cells cultured for 3 days in the absence or presence of PEG-rHuMGDF (reviewed in Ref. 12).

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**Fig. 5. Degradation of Cyclin A in cell extracts derived from Y10/L8057 cells.** A, Western blots of cell extracts derived from Y10/L8057 cells cultured for different days in IMDM medium containing 10% FCS alone or supplemented with 50 nM PMA, as indicated, using anti-cyclin A. B, cell extracts derived from Y10/L8057 cells cultured for 3 days without (lane 1) or with 50 nM PMA (lane 2) or with 50 ng/ml PEG-rHuMGDF (lane 3), were incubated at 30 °C for 30 min in the presence of 1 mM ATP and in the absence or presence of recombinant cyclin A (rec-cyclin A) as indicated. The reactions were then subjected to Western blot analysis using anti-cyclin A antibody. The data are representative of two experiments.

**Fig. 6. Degradation of recombinant cyclin B by extracts derived from polyploid and diploid MegT cells.** A, recombinant cyclin B1 was incubated for 30 min at 30 °C in the presence or absence of ATP, as indicated, with cell extracts derived from MegT cells cultured at 34 °C (proliferating cells) (lane 1), from adhering MegT cells cultured at 39.5 °C (proliferating cells) (lane 2), or from MegT cells in suspension cultured at 39.5 °C and in which T antigen is inactivated (polyploidizing cells) (lane 3). The reactions were subjected to Western blotting using a monoclonal antibody to cyclin B1. Incubation of recombinant cyclin B1 alone, in the absence of cell extract, is shown in lane C. 5–10-s exposure of the blot was sufficient for detecting the bands by the ECL system, while prolonged exposure did not yield additional bands. The data are representative of four experiments. B, the blot in panel A was briefly stained with Ponceau S to confirm equal loading of protein in each lane. C, Western blot analysis of a filter loaded with samples as in panel A, using a polyclonal antibody to cyclin B1. To detect bands by the ECL method, the exposure of x-ray film to the membrane was carried out for 20 min.
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TABLE II
DNA synthesis in proliferating and polyploidizing Y10/L8057 cells

| Days in culture | [3H]Thymidine incorporation |
|----------------|-----------------------------|
|                | Control | PMA          |
| 2              | 3.9     | 4.9          |
| 3              | 3.7     | 7.3          |

* cpm/cell × 10^-4.

Under our liquid culture conditions, PEG-rHuMGDF did indeed increase the frequency and ploidy of megakaryocytes (Table II). A cellular extract prepared from PEG-rHuMGDF-treated bone marrow cells degraded recombinant cyclin B more efficiently than extract from diploid bone marrow cells (Fig. 7). This was accompanied by a large shift of cyclin B to high molecular weight forms. It is technically impossible at this stage to purify significant quantities of proliferating, small megakaryocytes from primary bone marrow, thus preventing us from comparing the extent of cyclin B degradation in diploid versus polyploid megakaryocytes. We were, however, able to obtain a preparation of highly enriched polyploid megakaryocytes from bone marrow cells cultured with PEG-rHuMGDF (Fig. 8A). As shown in Fig. 8B, the rate of degradation of recombinant cyclin B1 by an extract derived from large megakaryocytes is significantly higher than the rate of degradation by diploid bone marrow cells. We realize that these results, although indicative, do not demonstrate conclusively that polyploidizing primary megakaryocytes display enhanced activity of the ubiquitin-proteasome pathway as compared with diploid megakaryocytes.

Megakaryocyte-induced Degradation of Cyclin B Depends on the Ubiquitin-conjugating Enzyme Ubc4—It has been reported that cyclin B degradation in other systems is mediated via the ubiquitin-conjugating enzyme Ubc4 (4). We thus used a mutated form of Ubc4 in which cysteine 85 was replaced by serine. Such a protein was shown to act in vitro as a dominant negative mutant (28). Our data indicated that mutated Ubc4, but not mutated Ubc2 protein, slowed the rate of degradation of recombinant cyclin B by purified megakaryocytes (Fig. 9), indicating that Ubc4 mediates cyclin B degradation in this cell type.

DISCUSSION

In some systems investigated, the lack of cyclin B1 alone is sufficient to drive endoreduplication. Endoreduplication in some Drosophila cell types is indeed associated with the lack of cyclin B1 (33). Also, the metaphase II arrest in mouse oocytes is controlled through destruction of cyclin B1 (34). Certain treatments, such as with 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 (35–37). We found previously that polyploidization in megakaryocytic cell lines is associated with reduced levels of cyclin B1 protein, but not of cyclin A or of cyclin B1 mRNA, as compared with the levels in diploid cells (9, 10). In this context, it is important to note that although cyclin B is important for the G2/M transition, low levels of cyclin B may also be needed for the S phase of yeast cell cycles (16, 38). In the current study, we found that cyclin B1 half-life in polyploidizing cells is decreased, as compared with that in proliferating cells. We
thus explored the possibility that a reduced level of cyclin B1 during endomitosis reflects an enhanced ability of these cells to degrade this cyclin, since this may result in abrogated mitosis (13). We found that polyploidization in the megakaryocytic cell lines MegT and Y10/L8057 or in primary megakaryocytes was associated with an enhanced ability of the cells to degrade recombinant cyclin B1 protein, as compared with the diploid proliferating megakaryocytic cell lines or nonmegakaryocytic bone marrow cells, respectively. Also, an extract of total bone marrow cells treated with the ploidy-promoting factor, the c-Mpl ligand, exhibited an augmented ability to degrade cyclin B, as compared with nontreated bone marrow cells. This proteolytic process displayed all of the hallmarks of a ubiquitin-dependent degradation (39), such as formation of high molecular weight complexes of cyclin, dependence on ATP, and competition by the mutated form of the ubiquitin-conjugating enzyme Ubc4.

Many components are involved in ubiquitination and degradation of cyclin B. Cyclin B ubiquitination is regulated via the ubiquitin-conjugating enzymes Ubc4 (4) and UbcX/Ubc10 (7, 40), and on ubiquitin ligase (E3) activity, the latter associated with a complex of proteins termed the anaphase-promoting complex (APC) or cyclosome (2, 41, 42). Together with the ubiquitin-activating enzyme, these components promote polyubiquitination of cyclin B, which targets the protein to proteolysis by the 26 S proteasome. Besides Cdc16 and Cdc27, the identities of the components of APC in mammalian cells remain to be explored. The APC determines the specificity of degradation of different cyclins. King and colleagues (4) concluded that different cyclins are recognized by different components of the APC. Since cyclin A levels are elevated during the G2/S phase of the mitotic or endomitotic cell cycles in megakaryocytes (10) and since our current study demonstrates that polyploidizing megakaryocytes do not display an enhanced ability to degrade recombinant cyclin A, it is reasonable to assume that a cyclin B1-specific component of the APC may be up-regulated during polyploidization. Future identification of the APC components will allow a further examination of this process, and one may speculate that ploidy-promoting factors may affect the expression of such an APC-specific component.

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