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Separation of Cells at Different Times Within G2 and Mitosis by Cyclin B1 Flow Cytometry

R.J. Widrow,[1,*] Peter S. Rabinovitch,[2] Keuna Cho,[4] and Charles D. Laird[3,4]

[1]Molecular and Cellular Biology Program and Program in Molecular Medicine, Fred Hutchinson Cancer Research Center, Seattle, Washington
[2]Department of Pathology, University of Washington, Seattle, Washington
[3]Departments of Zoology and Genetics, University of Washington, Seattle, Washington
[4]Program in Molecular Medicine, Fred Hutchinson Cancer Research Center, Seattle, Washington

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Multivariate flow cytometry using specific cyclin proteins and DNA content can identify cell populations at different points within the cell cycle. Quantification of cyclin B1 and DNA content reveals that cells with high levels of cyclin B1 predominantly have a 4C DNA content and are therefore in G2 or mitosis. We have examined whether separation of cells by levels of cyclin B1 could be used to discriminate cells at discrete times within these phases. Post-replicative cells progressively enter into fractions with higher levels of cyclin B1, indicating that this protein can be used as a marker of time in G2. Furthermore, cells in particular phases of mitosis can be greatly enriched by separation based on cyclin B1 levels. This method can thus be used to isolate cells at specific times within G2 and mitosis, periods of the cell cycle that have been difficult to study by cell fractionation. Cytometry 27:250–254, 1997. © 1997 Wiley-Liss, Inc.

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Different phases of the mammalian cell cycle can be assessed by various means, such as DNA content and acridine orange (1). Distinguishing populations of cells at progressive times within a given phase has, however, proved more difficult. More traditional approaches have usually involved prior cell synchronization by biochemical or mechanical techniques. It has been shown, however, that the expression patterns of several cyclin proteins are dramatically altered in cells that have been synchronized, indicating that normal cell cycle progression may be further perturbed (5). Other methods have involved the use of specific cell cycle markers such as Ki-67 and PCNA (7). Multivariate flow cytometric analysis of the expression of cyclin proteins and DNA content has been used to indicate additional landmarks for cell cycle position [reviewed in (4)]. Differential expression of cyclins A and B1 allow discrimination between cells in G2 and mitosis (6). This technique has also been used to discriminate endoreduplicated G1 cells from G2 cells with the same DNA content (3).

Cyclin B1 is a member of the mitotic class of cyclins that regulate entry into mitosis. This protein forms complexes with the serine/threonine kinase $p34^{cdc2}$ forming the M-phase promoting factor (MPF). Cyclin B1 accumulates during late S and G2, peaking in mitosis (8). The levels of cyclin B1 fall precipitously during the metaphase/anaphase transition (9). Sherwood et al. (10) have shown by flow cytometry that cyclin B1 levels rise abruptly as cells obtain a 4C DNA content. Furthermore, the 4C population of cells demonstrates heterogeneity in cyclin B1 levels. These data suggested that the level of cyclin B1 could potentially distinguish cells at different times in G2 and mitosis.

In this report, we demonstrate that multivariate flow cytometry using cyclin B1 and DNA content can be used to separate cells in different parts of G2 and mitosis. This technique will be useful to examine the timing of cellular events within these phases.

MATERIALS AND METHODS

Reagents

RPMI 1640, phosphate buffered saline (PBS) and fetal bovine serum (FBS) were purchased from Hyclone Laboratories Inc. (Logan, UT). Normal goat serum (NGS) was purchased from Vector Laboratories Inc. (Burlingame, CA). Bovine serum albumin, Ribonuclease A (RNase A), and trichloroacetic acid (TCA) were purchased from

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*Correspondence to: R.J. Widrow, 1124 Columbia St., C3-168, Fred Hutchinson Cancer Research Center, Seattle, WA 98104.
E-mail: rjwidrow@U.washington.edu

Sigma. 4′,6-Diamidino-2-phenylindole (DAPI) was purchased from Accurate Chemical and Scientific Corp. (Westbury, NY).

Cell Culture

The human lymphoblastoid cell line FF was derived from an adult male and established with EBV-transformed peripheral blood lymphocytes. Cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum. Prior to use, cells were suspended in fresh media at 5×10^5 cells/ml at least 12 h prior to use to ensure asynchronous exponential growth.

Tritiated Thymidine Labeling

^{3}H-methyl-thymidine was added at a final concentration of 5 μCi/ml.

Cell Fixation and Staining

A modified version of a method developed by C. Nillson (University of Washington) was followed (unpublished). Cells were centrifuged for 7 min at 1,000 rpm. (All subsequent centrifugation steps were similarly carried out.) Medium was aspirated and the cell pellet was washed once in PBS. Cells were resuspended in 1 ml PBS and forcefully pipetted into 5 ml of ice-cold 80% ethanol. This alcohol fixation was carried out for 2 hours at −20°C. Cells were then pelleted and washed once with 5 ml of 1% BSA in PBS. After centrifugation, cells were resuspended in 1 ml of 1% BSA and 0.25% triton X-100 in PBS, and placed on ice for 5 min. After the addition of 5 ml of 1% BSA in PBS, cells were repelleted and suspended in 200 μl of 1% BSA and 10% NGS in PBS. Cyclin antibody with a FITC conjugate (clone GNS-1; Pharmingen, San Diego, CA) was added at 0.5 μg per sample and left overnight at 4°C in the dark. All subsequent steps were carried out under yellow light. At least 45 min prior to flow analysis, 1 ml of 1% BSA in PBS was added to each sample. The cells were pelleted and resuspended in 300 μl of staining solution (PBS containing 1% BSA, 1 μg/ml RNase A (Sigma) and 10 μg/ml DAPI (Accurate Chemical and Scientific Corp.)) and incubated for 30 min at room temperature. Before flow analysis, 700 μl of 1% BSA in PBS was added to each sample.

Flow Cytometry

Flow cytometry was done on a FACS Vantage flow cytometer (Becton Dickinson). FITC was excited with a 488 nm laser and FITC data were collected through a 530 nm band pass filter. A multiline UV laser was used to excite DAPI. The DAPI data were collected through a 424 nm band pass filter. Data were acquired and analyzed in CellQuest (Becton Dickinson).

Scintillation Counting

Cells were collected from each of the 5 fractions G1, S, low-cyclin B1, mid-cyclin B1, and high-cyclin B1 (Fig. 1). Between 1000 and 2500 cells from each fraction were sorted onto separate GF/C filters (Whatman) and placed in a scintillation vial with 4 ml of Ecolite scintillation fluid (ICN). ^{3}H-thymidine was then measured in a scintillation counter. All of the detectable ^{3}H counts in washed and fixed cells were determined to be due to incorporated label (unpublished observations), and therefore TCA precipitation of the genomic DNA was not routinely done. Counts per minute for each sample were then calculated per 2500 cells after adjusting for background levels.

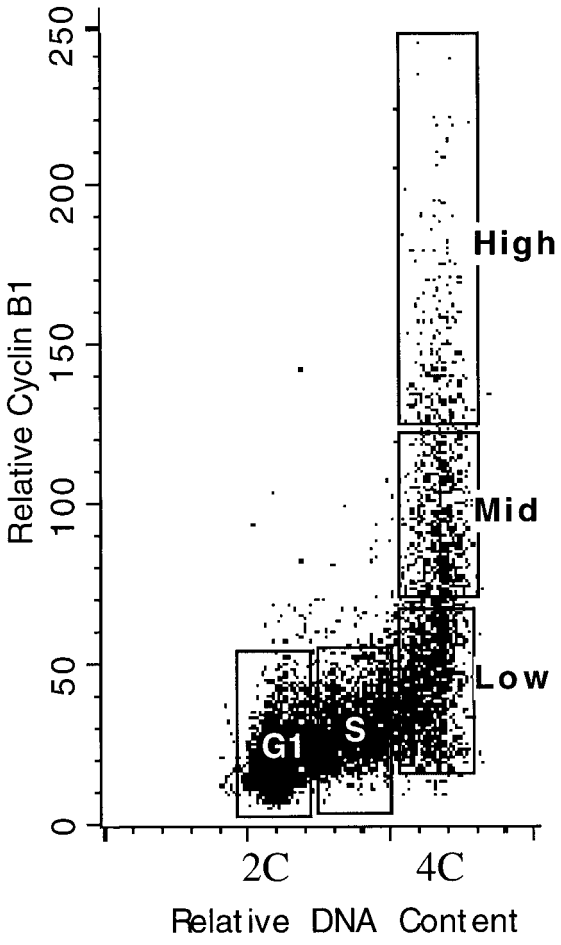

FIG. 1. Multivariate flow cytometric analysis of DNA and cyclin B1. Cells were simultaneously stained with DAPI (X-axis) and a FITC-conjugated anti-cyclin B1 antibody (Y-axis). The sorting regions represent the cell populations analyzed in subsequent experiments: G1, S, and 3 fractions of G2 + M (low, mid- and high-cyclin B1).

Immunofluorescence Microscopy

About 1000 cells from each of the 5 fractions described above were sorted directly onto microscope slides. Four μl of Dabco in glycerol (1:4) were then added before applying a glass coverslip and sealing with nail polish. For each fraction at least 100 random cells were scored for nuclear morphology as detected by DAPI fluorescence using a Zeiss Axiophot Photomicroscope. Images for Figure 4 were collected on a BioRad MRC600 scanning laser confocal microscope mounted on a Nikon Optiphot. Data were collected using a Nikon 40× n.a. dry objective. Full-frame (786 × 512) 8-bit images were collected for analysis and overlaid in 24-bit RGB using Adobe Photoshop v3.0.

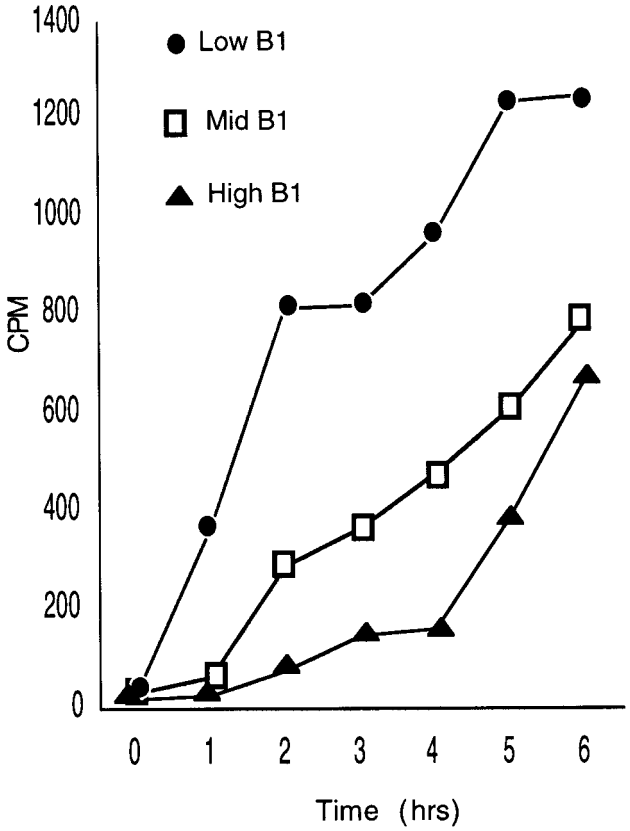

FIG. 2. Cyclin B1 levels can discriminate cells at successive times in G2. Asynchronously growing cells were labeled with ^{3}H-methyl thymidine. For each time point, cells were sorted into fractions as shown in Fig. 1 and assayed for the level of radioactivity (counts per minute).

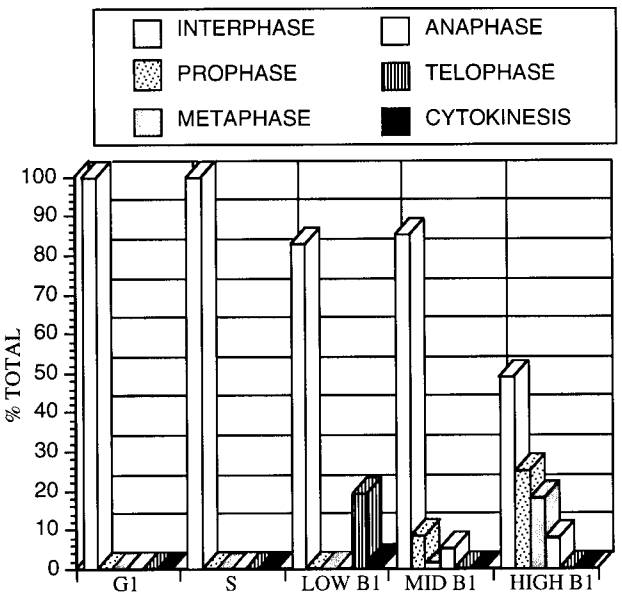

FIG. 3. Enrichment for cells in different stages of mitosis by cyclin B1. Cells from the five fractions previously described were sorted onto separate slides. The percentage of cells in different phases of mitosis in each fraction was determined by scoring cells on the basis of nuclear morphology as detected by DAPI fluorescence.

RESULTS

Cyclin B1 Expression in G2

Bivariate flow cytometric analysis shows that cyclin B1 accumulation occurs primarily in cells with a 4C DNA content (Fig. 1). The maximal cyclin B1 expression is approximately 5-fold higher than both the level of cyclin B1 detected in G1 cells, and the nonspecific fluorescence detected using a goat-anti mouse IgG (Fig. 1, data not shown). To determine if cyclin B1 could discriminate cells at different times in G2, cells were tracked in the following manner: asynchronously growing cells were labeled with ^{3}H-thymidine for up to 6 h; at various timepoints after labeling, cells from G1, S, and 3 fractions of G2 + M (low, mid-, and high-cyclin B1) were sorted on the basis of DNA content (DAPI fluorescence) and cyclin B1 content (FITC fluorescence). Each fraction was then assayed by scintillation counting for the level of incorporated ^{3}H-thymidine per cell.

If, as we hypothesized, the level of cyclin B1 staining in G2 cells is heterogeneous principally because the intensity of cyclin B1 expression increases as cells transit through G2, then radiolabeled cells should appear first in the 4C cell populations with low cyclin B1, and in higher cyclin B1 fractions over time (Fig. 2). After 1 h, significant label was detected only in the low-cyclin B1 fraction. This indicates that as cells exit S-phase, they reside in G2 before substantial cyclin B1 accumulation, consistent with previous reports (10). Half-maximal levels of label appeared at successively later times in the low (1.8 h), mid (3.2 h), and high (4.8 h) compartments of cyclin B1. The appearance of radiolabeled cells over time in fractions with successively higher levels of cyclin B1 confirms that the rise of this protein can be used as a marker of G2 progression. Consequently, separation of cells in this manner can isolate populations that have been in G2 for discrete times.

Cyclin B1 Expression During Mitosis

The levels of cyclin B1 have been shown to increase late in S-phase and peak in mitosis during the metaphase/anaphase transition (9). Cyclin B1 is then rapidly degraded through a ubiquitin-mediated pathway (2). Since the rise and fall of cyclin B1 occurs in cells with a 4C DNA content, an individual cell with a given cyclin B1 level may be in either G2 or mitosis. Hence the cell population within a range of cyclin B1 levels is heterogenous with respect to cell cycle position. To determine the extent of this heterogeneity, we separated cells on the basis of DNA content and cyclin B1 levels as described earlier and scored for nuclear morphology. Although mitotic cells were found in all 3 cyclin B1 fractions of 4C cells, there was substantial enrichment of cells during different phases of mitosis among the sorted fractions (Fig. 3). Cells with a 4C DNA content and an interphase nuclear morphology, i.e., G2 cells, were found in all 3 fractions. From the

^{3}H-thymidine–labeling experiments, it can be inferred that these represent G2 cells at successively later times.

Prophase cells were found in both the mid- and high-cyclin B1 fractions, with more enrichment in the later. Similar results were found for metaphase cells, except that these cells were even more enriched in the high-cyclin B1 fraction. Anaphase cells were found predominately in the mid- and high-cyclin B1 fractions. Finally, telophase cells, as well as cells undergoing cytokinesis, were found only in the low-cyclin B1 fraction. These data suggest that cyclin B1 levels accumulate substantially before cells enter mitosis, peak during metaphase, and decline rapidly as they proceed through anaphase. These results confirm other reports that have shown that the level of cyclin B1 falls to G1 levels by telophase (9).

The cellular localization of cyclin B1 was examined in cells from each of the 5 fractions. The protein surrounds the nucleus during late S and G2 (Fig. 4A–D). Only after the cells had entered prophase is cyclin B1 prominent within the nucleus (Fig. 4C–F). By telophase, the levels of cyclin B1 were too low to detect (Fig. 4G,H). These changes throughout the cell cycle are consistent with previous results (9).

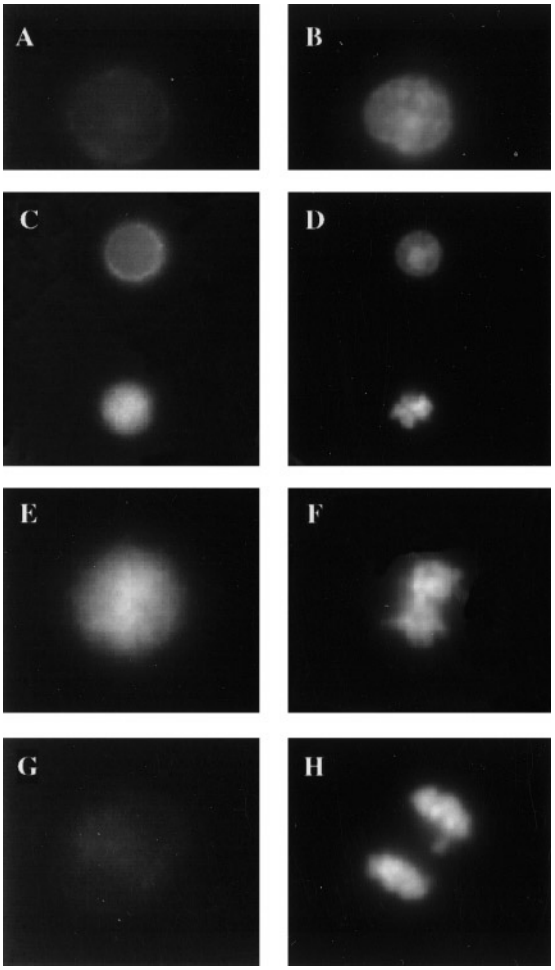

FIG. 4. Localization of cyclin B1 throughout the cell cycle. Sorted cells from each cyclin B1 fraction were examined by immunofluorescent microscopy. (**A**) Interphase cells from the low–cyclin B1 fraction show a predominantly cytoplasmic localization of cyclin B1. (**B**) Same cell as in (A), stained with DAPI. (**C**) Cells in the mid-cyclin-B1 fraction can be late G2 (**top**) or mitotic (early anaphase, **bottom**). (**D**) Same cells as in (C) stained with DAPI. (**E**) Only after cells enter mitosis is cyclin B1 detected primarily within the nucleus illustrated by this metaphase cell. (**F**) DAPI staining of the same cell in (E). (**G**) Late anaphase and telophase cells have low levels of cyclin B1 protein. (**H**) DAPI staining of the cell in (G). Images in (C) and (D) are reduced in magnification compared with other images to include both cells.

DISCUSSION

In this report, we have demonstrated that the separation of cells simultaneously on the basis of DNA content and cyclin B1 content can yield enriched cell populations representing different times within G2 and mitosis. In particular, labeled cells entering G2 progress successively though the low, mid, and high levels of cyclin B1. Additionally, analysis of DAPI and cyclin B1–fractionated cells confirmed that cyclin B1 accumulates before entry into mitosis, peaks during metaphase, and is degraded in anaphase. The success of our protocol indicates that cyclin B1 is a sensitive molecular indicator of cell cycle position. Nevertheless, there was not absolute synchrony within the population with respect to cyclin B1 levels. Consequently, cell separation in this manner provides enrichment for various cell cycle populations, rather than an absolute purification of a homogenous population. Since cyclin B1 levels both rise and fall while cells have a 4C DNA content, cells represented by a given cyclin B1 level can be either in G2 or mitosis (for example, see Fig. 4B). It has been shown that differential levels of cyclin A and B1 can be used to differentiate G2 cells from mitotic cells (6). Hence a combination of these techniques could be used to isolate homogenous populations of cells in successive periods of G2 and mitosis.

The G2 phase has traditionally been defined as the period following DNA replication preceding the onset of mitosis. This definition gives the false impression that the cell is in a static state during this phase. In fact, during G2, cells have to ensure that DNA replication has been completed, as well as to prepare adequately for entry into mitosis. Isolation of cell populations at different points within G2 will allow further analysis of the timing and control of these events.

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