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THE PHOSPHORYLATION OF RETINOBLASTOMA GENE PRODUCT IN HUMAN MYELOID LEUKEMIA CELLS DURING THE CELL CYCLE

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SUMMARY: Counterflow centrifugal elutriation and immunoblotting techniques were used to study the expression of the retinoblastoma (RB) gene during the cell cycle of BV173 chronic myeloid leukemia (CML) cells. Our data showed that Rb protein started to be phosphorylated at early G1 phase, became hyperphosphorylated when cells progressed to late G1 and S phases during cell cycle, and remained hyperphosphorylated throughout S and G2/M phases. Our data suggest that Rb phosphorylation starts at a more distal point to the G1/S phase boundary in human myeloid leukemia BV173 cells rather than at a point more proximal to the G1/S boundary, as seen in HeLa cells.

The retinoblastoma tumor suppressor gene product (Rb) is involved in cellular proliferation and differentiation. Inactivation of the RB gene by deletions or point mutations has been associated with the development of retinoblastoma, small cell lung carcinoma, breast cancer, osteosarcoma, and bladder carcinoma [1-7]. The introduction of the normal RB gene into retinoblastoma and osteosarcoma cell lines resulted in a suppressed neoplastic phenotype, indicating the tumor-suppression function of the RB gene [8].

The mechanism through which the RB gene regulates cellular proliferation is not fully understood. Studies of Rb regulation during the cell cycle have shown that phosphorylation of Rb is cell cycle regulated [9-12]. The underphosphorylated form of Rb (Rb110), which is associated with G0\G1 phase, may have the growth-suppression function. The hyperphosphorylated Rb (Rb116), which is associated with S and G2\M phase, may have lost the growth-suppression function and gained a growth-stimulatory function.

It has been suggested that phosphorylation of the Rb protein starts in late G1 and early S phase or at the G1/S boundary in HeLa cells [10,12]. We studied the expression of the Rb protein during the cell cycle of human chronic myloid leukemia (CML) BV173 cells [13] and found that phosphorylation of Rb occurred relatively early in G1. By late G1, most of the Rb protein exists in the hyperphosphorylated form, suggesting that the schedule of Rb phosphorylation in the cell cycle may be different in different cell types.

Material and Methods

Immunoblotting: Total proteins were extracted by boiling 10^7 cells in 1 ml of lysis buffer (0.125 M Tris, pH 6.8, 1% sodium dodecyl sulfate (SDS), 2% ß-mercaptoethanol, and 5% glycerol) for 4-5 min, followed by vortexing. The protein extracts equivalent to 5 x 10^5 cells were analyzed by 10% SDS-

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polyacrylamide gel electrophoresis (PAGE). Proteins were then transferred to Immobilon PVDF membrane (Millipore) according to the manufacturer's instruction. The immunoblotting was done as described [14]. The levels of Rb, proliferating cell nuclear antigen (PCNA) and actin were detected by primary antibodies against Rb (Triton Bioscience Inc.), PCNA (Boehringer Mannheim), and actin (Oncogene Science). The blots were then incubated with a [^{125}I]-labeled secondary antibody (Amersham Corp.), followed by autoradiography.

Cell Elutriation: Exponentially growing BV173 cells were resuspended in Hank's buffer supplemented with 4% fetal calf serum (FCS). Cells were loaded onto the Beckman JE-10X elutriator with a rotor speed of 3400 rpm and temperature of 8°C. Eighteen fractions were collected by increasing the flow rate stepwise from 24 ml/min to 83 ml/min. Thirty-five milliliters were collected in the first two fractions, and 50 ml in each of the remaining fractions. Cells in each fraction were analyzed for their size and enumerated by a Coulter counter analysis.

Flow Cytometry: Cells (10^6) from each fraction were fixed by gradual addition of cold ethanol (-20°C) while vortexing. After one day at 4°C, the fixed cells were washed with phosphate buffered saline (PBS), then deproteinized with 1 ml of 0.04% pancreatic pepsin (Sigma) w/v in 0.01N HCl for 20 min at room temperature under gentle shaking. Cells were next washed twice with 3 volumes of PBS at pH 7.2, then resuspended in 1 ml of propidium iodide (PI) at 10 μg/ml in PBS containing 0.5% Tween 20 (Sigma) and 400 units of pancreatic RNase (Sigma). After 2 h of incubation in the dark, cells were analyzed in a FACSCAN flow cytometor (Becton Dickinson) equipped with a doublet discriminator. Cell cycle distribution for each sample was analyzed with the Cellfit software (Becton Dickinson) after gating out all cell doublets and debris.

Results

Exponentially growing BV173 cells were separated into 18 fractions enriched in G1, S, or G2/M phases by counterflow centrifugal elutriation [15]. The cell cycle status of cells in each fraction, except fractions 1 and 2, was determined by flow cytometry, as presented in Table 1 and Figure 1. In fractions 1 and 2, the number of cells did not permit the cell cycle analysis but were assumed to be composed of early G1-phase cells. Cells at G1 phase predominated in early fractions, then declined at later fractions, while cells at S and G2/M phases increased.

The elutriation samples were analyzed for Rb protein by Western blotting using Rb-specific monoclonal antibody. We showed (see Fig. 2) that the levels of Rb protein were very low in early G1 phase (Fractions 1,2), and the Rb protein was in the underphosphorylated form (110 kDa). The levels of Rb protein then increased, and the Rb protein became phosphorylated, as indicated by the increased molecular mass in fractions 3, 4, and 5, in which the majority of cells (85%) were in G1. Furthermore, in fractions 5, 6, and 7, the percentage of cells in S phase is 15-18%; however, the levels of hyperphosphorylated Rb (Rb116) was already equal to or more than that of underphosphorylated Rb. The levels of Rb protein increased two- to threefold as cells progressed into S phase and slightly declined as cells moved into G2 and M phases (Fig 2, lanes 15-18). In fractions 13 and 14, 66.5% and 64.8% of cells were in S phase, respectively, 23.1% and 16.4% of cells were in G1, respectively, and almost all Rb protein was converted to the hyperphosphorylated isoform (116 kDa) in these fractions, suggesting that Rb exists in hyperphosphorylated form in cells of late G1 phase.

Our results demonstrate that in CML BV173 cells, Rb is progressively phosphorylated as the cell progresses from early G1 to late G1. By the time the cells reach late G1, almost all the Rb protein is hyperphosphorylated. Levels of the 116-kDa isoform of Rb declined during G2 and M phase (see Fractions 16-18 in Fig. 2); conversion to underphosphorylated Rb was not seen.

TABLE 1. The peak size and cell cycle composition of cells in fractions of elutriated BV173 cells

Fraction	Peak Size (μM)	G1 Phase %	S Phase %	G2 + M Phase %
1	-	-	-	-
2	-	-	-	-
3	8.17	94.6	4.4	0.0
4	8.27	95.5	4.5	0.0
5	8.65	84.9	15.1	0.0
6	8.91	84.9	15.1	0.0
7	9.08	81.8	18.2	0.0
8	9.24	67.6	30.8	1.6
9	9.40	61.7	37.9	0.4
10	9.62	48.9	48.1	3.0
11	9.69	39.5	56.5	4.0
12	9.90	32.2	63.1	4.7
13	10.23	23.1	66.5	10.4
14	10.54	16.3	64.8	18.9
15	10.95	15.7	57.0	27.3
16	11.06	11.1	49.2	39.7
17	11.49	11.6	35.9	52.5
18	11.69	16.5	40.0	43.5

We reblotted the same filter with an anti-actin antibody to monitor the amount of protein in each lane (Fig. 2B). Comparable amounts of protein are shown to be in each lane. An anti-PCNA antibody was used to analyze the levels of PCNA during the cell cycle. PCNA is an accessary factor to DNA polymerase-δ. It has a basal level in G1 phase, increases when cells progress into S phase, and peaks at S phase [16,17]. This expression pattern of PCNA is also shown in BV173 cells during the cell cycle (Fig. 2C).

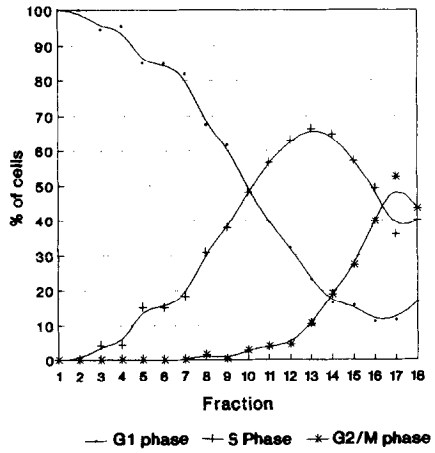

Figure 1. The cell cycle profile of different fractions of elutriated BV173 cells.

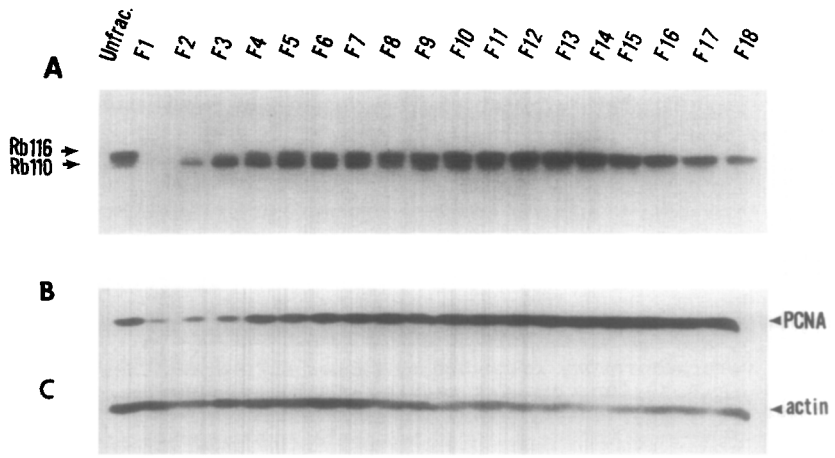

Figure 2. Protein was extracted from 5 x 10^5 cells of unfractionated cells (lane 1) and cells of each fractions. The levels of Rb, actin, and PCNA were analyzed by western blotting using anti-Rb, anti-actin, and anti-PCNA antibodies.

Discussion

Our results demonstrate that in CML BV173 cells, Rb is progressively phosphorylated as the cells progress from early G1 to late G1. In late G1, the great majority of Rb protein is in a hyperphosphorylated form (Rb116), suggesting that in BV173 cells, the kinase that phosphorylates Rb is activated early during the cell cycle. This pattern is different from that of HeLa cells, in which Rb phosphorylation occurs at a more proximal position to the G1/S boundary, and substantial phosphorylation can be observed in the elutriated fractions of HeLa cells in which 30-40% S-phase has been reached [10,12]. It has also been shown that in phorbol 12-myristate 13-acetate-stimulated T lymphocytes, Rb phosphorylation occurs well before DNA synthesis [18], implying that Rb phosphorylation is not a proximal event regulating G1 to S transition.

These observations indicate that the kinase phosphorylating Rb may be activated at different time points, which are either distal or proximal to the beginning of S phase in different cells. It is conceivable that the timing is related to the position of the restriction point in G1 phase during the cell cycle. We have observed that BV173 cells are highly proliferative; its growth was not hampered by removal of FCS from the medium. However, removal of FCS greatly decreased the growth of HeLa cells. It seems that the early schedule of Rb phosphorylation correlates with the high intrinsic potential for proliferation of the cells.

Recently, a 6- to 10-h window prior to DNA synthesis was identified in osteosarcoma Saos-2 cells during which injection of Rb protein inhibited the cells from entering S phase [19]. Injection of Rb into cells outside of this window had no effect on the progression of cells into S phase. It is conceivable that this window represents the period of kinase activation and Rb phosphorylation. After this window in the cell cycle, the kinase system may become highly activated so that the injected Rb would rapidly become phosphorylated and therefore lose the ability to suppress cellular proliferation. It is very likely that different cells have different windows and different

critical points in the G1 phase. In BV173 cells, Saos-2 cells, and phorbol 12-myristate 13-acetate- stimulated lymphocytes, the window is probably more distal to the onset of DNA synthesis, while in HeLa cells, the window is probably more proximal to the onset of DNA synthesis.

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