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Expression of G1-Phase Cell Cycle Genes during Hematopoietic Lineage

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Characterization of proteins that control the passage through the G_1 phase of the cell cycle is of particular interest because virtually all stimuli regulating cell proliferation or differentiation act primarily during this phase. We have analyzed the G_1 phase proteic machinery, including cyclin D types, cyclin-dependent kinases (CDKs) and CDK inhibitors, of cell populations obtained at different stages of hematopoietic cell lineage. In particular, five cellular phenotypes, namely CD34+ cells (which contain stem cells), BFU-E, CFU-E, CFU-GM and peripheral lymphocytes were studied as representatives of distinct differentiation pathways. The results obtained indicated that all the cellular preparations express cyclin D2 and D3, while cyclin D1, which is the major cyclin D occurring in mesenchimal tissues, is not expressed. Moreover, CDK6 (but not CDK4) was detectable in all the populations investigated. Among the CDK inhibitors studied, $p18^{INK4C}$ and $p19^{INK4D}$ signals were clearly evidentiable in the various cell types. Interestingly, high levels of $p15^{INK4B}$, a putative tumor suppressor protein, were detectable expecially in granulocyte-monocyte precursors. Our results indicate that a specific hematopoietic G_1 phase machinery occurs, which is conserved during the various steps of the different maturation processes.

The development of a multicellular organism and the process of cell lineage require the coordinate expression of genes involved in cell proliferation, arrest and differentiation. Generally, stimuli determining whether cells continue to grow or cease dividing and differentiate appear to operate mainly during G1 phase of the cell division cycle (1).

The molecular mechanisms which allow the progression through G_0 and G_1 phases as well as S phase entry are quite complex, although some biochemical events have been definitely characterized. In particular, cell growth depends upon several cyclin-dependent kinase (CDK) activities, which are regulated by phase-specific cyclins and different CDK inhibitors.

Two distinct, but remarkably homologue, mid-to-late G_1 phase CDKs have been identified (CDK4 and CDK6) (2-4) which are activated by binding with one of the members of cyclin D family (cyclin D1, D2 and D3) (2-4). Two families of CDK inhibitors are known at present, one includes proteins belonging to the so-called $p16^{INK4A}$ family (i.e. $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$) (5-9), while the second, the $p21^{CIP1}$ family, contains $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$ (10-12).

Tissue distribution of CDKs has been investigated in some instances, suggesting that CDK4 (but not CDK6) is expressed in the great majority of cell types (13), while cyclin D1 is the widest occurring cyclin D observable in mesenchimal tissues (14). A clear exception to this pattern is represented by peripheral lymphocytes which express a substantially different pattern of G_1 phase cell cycle machinery, in that CDK6 and cyclin D2 and D3 are the most important proteins (13, 15). The expression of the members of $p16^{INK4A}$ family has not been examined in details, although the scarce available information suggests that $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$ are almost ubiquitously distributed, while $p16^{INK4A}$ is more specifically expressed (7, 9, 16). Few data are also present on the expression of G_1 phase genes during the process of cell lineage and the only reports available described the differentiation of established cell lines (17, 18), which are generally obtained from malignant tissues and thus do not represent model of normal cellular differentiation.

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Abbreviations: CDK, cyclin-dependent kinase; PBS, phosphate buffered saline; PEB, PBS/5 mM EDTA/0.5% bovine serum albumin.

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In order to investigate the possible change of expression pattern of genes involved in the G_1 phase progression, we examined the cell growth machinery during the normal hematopoietic cell lineage. To avoid potential artifacts, related to the employed cellular model, we used freshly isolated hematopoietic early progenitors, which were subsequently induced to differentiate by the addition of specific stimulating factors. In particular, we analyzed CD34+ cells (which contain hematopoietic stem cells), BFU-E and CFU-E (as intermediate cell populations of erythropoietic lineage), CFU-GM (as granulo-monocyte progenitors) and peripheral lymphocytes. These cell preparations were studied by immunoblotting analyses using antibodies against several G_1 phase proteins. The observed pattern represents the first report on the characterization of a phase-specific cell cycle machinery during physiological cell lineage.

MATERIALS AND METHODS

Cell preparation. Bone marrow samples were diluted 1:4 with phosphate buffered saline (PBS), layered over Ficoll-Hypaque (1077 SD; Pharmacia, Uppsala, Sweden) and centrifuged at 1,500*g* for 30 min. Light-density mononuclear cells were collected and washed once in PBS and centrifuged as above. The pellet was washed again in PBS/5 mM EDTA/0.5% bovine serum albumin (PEB) and centrifuged. The pellet was resuspended in PEB at a concentration of 3×10^8 cells/ml. The cell suspension was then subjected to magnetic sorting as follow. Blocking and antibody reagents (QBEND 10) from the CD34 isolation kit (MACS, Miltenyl Biotec, Germany) were added simultaneously to the cell preparation and incubated at 4°C for 15 min. After this period, the cells were centrifuged and washed twice with PEB. Colloidal submicroscopic magnetic beads were then added to the cell suspension, incubated for 15 min at 4°C, pelletted by centrifugation and washed twice with PEB. The washed pellet was carefully resuspended in PEB and applied to a prefilled column. Cells which did not bind CD34 antibody passed through the column, while CD34+ cells were retained. The CD34+ cells were eluted by removing the column from the magnetic field and flushing the column with PEB. The magnetic separation was repeated on a second column. Purity of the separated cells was determined by flow citometry and was estimated higher than 90%.

CD34+ cells were cultured at a concentration of 25×10^3 cells/ml in Iscove's modified Dulbecco's medium (Life Technologies, MD, USA) plus 30% fetal calf serum. Erythroid progenitors were obtained after 7 (BFU-E) and 14 (CFU-E) days culture in presence of 5×10^{-5} M β-mercaptoethanol, 200 mM glutamine, 100 U/ml interleukin 3 (Genzyme, MA, USA), 2 U/ml erythropoietin (Cilag, AG, Switzerland), 20 ng/ml stem cell factor (Genzyme). Granulomonocyte progenitors (CFU-GM) were obtained from CD34+ by culturing the cells for 14 days in the presence of 100 U/ml interleukin 3 and 200 U/ml granulocyte-macrophage colony-stimulating factor (Sandoz, Switzerland). Peripheral blood lymphocytes were prepared as described in 19. All cell preparations were stored as pellet under liquid nitrogen until use.

Antibodies. $p16^{INK4A}$ antibodies were provided from PharMingen, San Diego, CA, USA (against the full protein) and Santa Cruz Biotechnologies, Santa Cruz, CA, USA (against a peptide corresponding to the amino acids 128-147 mapping at the C-terminus). An additional anti-$p16^{INK4A}$ serum (directed against the 16 C-terminal amino acids peptide) was prepared in our laboratory and purified by immunoaffinity. Rabbit polyclonal antibodies directed against human CDK4 and cyclin D1 were given by Drs G. Draetta and M. Pagano (Mitotix Inc., Boston, MA, USA) while an antiserum against CDK6 was kindly given by Dr. Gordon Peters, Imperial Cancer Research Fund Laboratories, London, UK. Antibodies against $p18^{INK4C}$ were a gift of Dr Y. Xiong, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA. $p15^{INK4B}$, $p19^{INK4D}$, cyclin D2, cyclin D3 and $p27^{Kip1}$ antisera were obtained from Santa Cruz.

Immunochemical methods. Cell extracts were prepared as described in 20. 50-200 μg of cell extracts were analyzed by SDS-PAGE employing a 15% acrylamide resolving gel, transferred to a nitrocellulose membrane and incubated with different antisera (20). The immunocomplexes were detected by the alkaline phosphatase method or by the ECL techniques as described in 20.

RESULTS AND DISCUSSION

In order to characterize the G_1 phase cell cycle machinery during cell lineage processes, we analyzed the differentiation of CD34+ cells towards erytroid (BFU-E and CFU-E) or monocyte-granulocytes (CFU-GM) progenitors. In addition, the results obtained were compared to those on peripheral blood lymphocytes. We selected cyclin D family, CDK4 and CDK6 and the CDK inhibitors belonging to the $p16^{INK4A}$ family as the most intriguing targets of investigation since: i) these proteins play their role in G_1 phase, which has a major function during the differentiation process and ii) they show a remarkable tissutal specificity opposed to other G_1 phase proteins (namely CDK2 and cyclin E) which are ubiquitously expressed (2-4).

It is to underline that the cell growth proteins were evidenced by direct immunoblotting analysis, avoiding the use of alternative techniques such as detection of specific mRNA or protein cell labelling (by ^{35}S-methionine) followed by immunoprecipitation. Such a choice is related to the possibility of creating experimental artifacts due to different half-lives of various mRNAs or to variability in the labelling rate of distinct proteins. Conversely, we analyzed directly cell extracts by sensitive immunochemical techniques employing, in some cases, different antibodies for an identical antigen.

The results of our investigation are reported in Table 1 and some examples of immunoblotting analyses are reported in Fig. 1. In all the cell types studied cyclin D1 was not (or scarcely) expressed, while signals of cyclin D2 and D3 were clearly observable. In particular, the D2 type seems to be abundant in erythroid and monocyte-granulocyte precursors, while high amounts of cyclin D3 were evidentiable in CD34+ and CFU-GM cells. CDK6 was clearly detectable in CD34+ cells and in all the other cell types, whilst CDK4 was evidentiable (as a faint signal) only in CFU-E. $p18^{INK4C}$ and $p19^{INK4D}$ appeared to be expressed in all the cell preparations analyzed. $p15^{INK4B}$ signals were observable in all the samples investigated, with a major intensity occurring in CFU-GM and a lower level in peripheral blood lymphocytes. Finally, $p16^{INK4A}$ band was observable, as a faint signal, solely in CFU-E, while none of the other cell types expressed this protein. In the case of $p16^{INK4A}$ we have used three different antisera, one

TABLE 1

G_1-Phase Cell Cycle Division Machinery of Human Hematopoietic Cells

Protein	CD34+	BFU-E	CFU-E	CFU-GM	Lymphocytes
Cyclin D1	–	–	+/–	–	–
Cyclin D2	+	++	+++	+++	+
Cyclin D3	+++	+	+	+++	+
CDK4	–	–	+/–	–	–
CDK6	+	+	+	+	+
$p16^{INK4A}$	–	–	+/–	–	–
$p15^{INK4B}$	++	++	++	+++	+
$p18^{INK4C}$	+	+	+	+	+
$p19^{INK4D}$	+	+	+	+	+
$p27^{Kip1}$	+	+	+	+	++++

Note. Protein levels were determined by immunoblotting as reported under Materials and Methods.

directed against the full protein and two against the C-terminus of this CDK inhibitor. In all these experiments only CFU-E cells showed to express $p16^{INK4A}$ protein. We have also analyzed our cell preparations for the occurrence of $p27^{kip1}$ protein. This choice is related to the important role played by the protein in the control of lymphocytes proliferation (21). The level of $p27^{kip1}$ protein was very high in peripheral lymphocytes, while lower but clearly detectable amounts of this CDK inhibitor were evidentiable in all the cell types analyzed.

A number of interesting considerations stems out from our results. First of all, the pattern described in lymphocytes, namely the occurrence of cyclin D2, cyclin D3 and CDK6 as major G_1 phase proteins, is observable in CD34+ cells as well as in all the analyzed hematopoietic differentiation steps. In other words, bone marrow early precursors express the same pattern as all the different (namely erythroid, lymphoid and granulomonocytic) lineages do. A partial exception to this picture is represented by the transition from the BFU-E->CFU-E, where we observed the expression of small amount of cyclin D1 and CDK4. Future studies are required to clarify if these proteins have any relevance in the erythrocytic maturation process. Two CDK inhibitors, namely $p18^{INK4C}$ and $p19^{INK4D}$, were expressed in all cell types, confirming the data available in literature which suggest their ubiquitously occurrence (7, 9). The absence of $p16^{INK4A}$ in the different cellular preparations confirms and extends the observation reported on lymphocytes and suggest a noticeable tissutal specificity for this CDK inhibitor. Moreover, the presence of small amounts of $p16^{INK4A}$ protein contemporaneously with the appearance of cyclinD1/CDK4 might suggest a coordinate expression of these proteins.

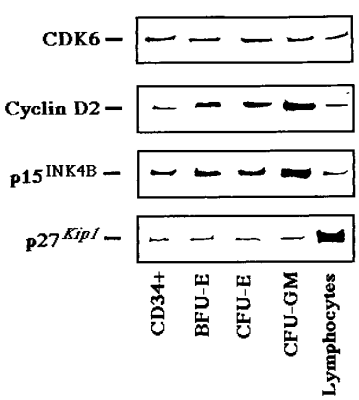

FIG. 1. Immunoblotting detection of CDK6, cyclin D2, $p15^{INK4B}$, and $p27^{Kip1}$ in CD34+, BFU-E, CFU-E, CFU-GM and peripheral lymphocytes. About 80 μg protein were separated by denaturing polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and incubated with the antibodies to the indicated proteins.

An important finding is the demonstrated occurrence of large amounts of $p15^{INK4B}$ in all the cell types examined, including CD34+ population and the progenitors of the different cell lineages. This result is particularly intriguing when compared to the absence of $p16^{INK4A}$ protein, since a coordinate expression of these two CDK inhibitors might be hypothesized. Indeed, several reports have demonstrated that these two genes, which map at about 20kb distance on a 9p21 chromosome locus and are thought of as important tumor suppressor genes, are homozygously codeleted in a tremendous number of cancers and, in particular, in childhood acute T-cell lymphoblastic leukemias (22, 23). Since $p15^{INK4B}$ gene is expressed in almost all the hematopoietic cell types investigated, it is possible that its inactivation is a key step in the development of hematologic malignancies.

In summary, our results demonstrated that the G_1 phase protein machinery is highly conserved from early bone marrow precursor cells throughout the various differentiative pathways. This is particularly important when compared to the finding that other mesenchimal cell types (fibroblasts, osteoblasts and chondrocytes) show a completely different G_1 phase machinery, characterized by high level of cyclin D1, CDK4 and $p16^{INK4A}$ proteins (24). Thus, the present study sug-

gests that the choice of G_1 phase machinery occurs very early during tissutal development and is mantained during the successive maturation pathways.

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REFERENCES

1. Pardee, A. B. (1989) *Science* **246,** 603–608.
2. Sherr, C. J. (1994) *Cell* **79,** 551–555.
3. Nurse, P. (1994) *Cell* **79,** 547–550.
4. Hunter, T., and Pines, J. (1994) *Cell* **79,** 573–582.
5. Serrano, M., Hannon, G. J., and Beach, D. (1993) *Nature* **366,** 122–126.
6. Hannon, G. J., and Beach, D. (1994) *Nature* **371,** 257–260.
7. Guan, K.-L., Jenkins, C. W., Li, Y., Nichols, M. A., Wu, X., O'Keefe, C. L., Matera, A. G., and Xiong, Y. (1994) *Genes Dev.* **8,** 2939–2952.
8. Chan, F. K. M., Zhang, J., Cheng, L., Shapiro, D. N., and Winoto, A. (1995) *Mol. Cell. Biol.* **15,** 2682–2688.
9. Hirai, H., Roussel, M. F., Kato, J.-K., Ashmun, R. A., and Sherr, C. J. (1995) *Mol. Cell. Biol.* **15,** 2672–2681.
10. Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993) *Nature* **366,** 701–704.
11. Toyoshima, H., and Hunter, T. (1994) *Cell* **78,** 64–74.
12. Lee, M. H., Reynisdottir, J., and Massaguè, J. (1995) *Genes Dev.* **9,** 639–649.
13. Tam, S. W., Theodoros, A. M., Shay, J. W., Draetta, G., and Pagano, M. (1994) *Oncogene* **9,** 2663–2674.
14. Lukas, J., Pagano, M., Staskova, Z., Draetta, G., and Bartek, J. (1994) *Oncogene* **9,** 707–718.
15. Meyerson, M., and Harlow, E. (1994) *Mol. Cell. Biol.* **14,** 2077–2086.
16. Quelle, D. E., Ashmun, R. A., Hannon, G. J., Remberger, P. A., Trono, D., Richter, K. H., Walker, C., Beach, D., Sherr, C. J., and Serrano, M. (1995) *Oncogene* **11,** 635–645.
17. Kiyoaka, M., Richon, V. M., Rifkind, R. A., and Marks, P. A. (1994) *Mol. Cell. Biol.* **14,** 7195–7203.
18. Ando, K., Ajchenbaum-Cymbalista, F., and Griffin, J. D. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 9571–9575.
19. Firpo, E. J., Koff, A., Solomon, M. J., and Roberts, J. M. (1994) *Mol. Cell. Biol.* **14,** 4889–4901.
20. Della Ragione, F., Russo, G. L., Oliva, A., Mastropietro, S., Mancini, A., Borrelli, A., Casero, R. A., Iolascon, A., and Zappia, V. (1995) *Oncogene* **10,** 827–833.
21. Nourse, J., Firpo, E., Flanagan, W. M., Coats, S., Polyak, K., Lee, M.-H., Massaguè, J., Crabtree, G. R., and Roberts, J. M. (1994) *Nature* **372,** 570–573.
22. Iolascon, A., Faienza, M. F., Coppola, B., Della Ragione, F., Santoro, N., and Schettini, A. (1995) *Brit. J. Haem.* **91,** 647–651.
23. Della Ragione, F., Mercurio, C., and Iolascon, A. (1995) *Haematologica* **80,** 557–568.
24. Tam, S. W., Shay, J. W., and Pagano, M. (1994) *Cancer Res.* **54,** 5816–5820.