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The Expression of $p18^{INK4}$ and $p27^{kip1}$ Cyclin-Dependent Kinase Inhibitors Is Regulated Differently During Human B Cell Differentiation

Nicolas Schrantz, Georges Edouard Beney, Marie Thérèse Auffredou, Marie Françoise Bourgeade, Gérald Lecha, and Aimé Vazquez

Cell cycle progression is under the control of cyclin-dependent kinases (cdks), the activity of which is dependent on the expression of specific cdk inhibitors. In this paper we report that the two cdk inhibitors, $p27^{kip1}$ and $p18^{INK4c}$, are differently expressed and control different steps of human B lymphocyte activation. Resting B cells contain large amounts of $p27^{kip1}$ and no $p18^{INK4c}$. In vitro stimulation by *Staphylococcus aureus* Cowan I strain or CD40 ligand associated with IL-10 and IL-2 induces a rapid decrease in $p27^{kip1}$ expression combined with cell cycle entry and progression. In contrast, in vitro Ig production correlates with specific expression of $p18^{INK4c}$ and early G1 arrest. This G1 arrest is associated with inhibition of cyclin D3/cdk6-mediated retinoblastoma protein phosphorylation by $p18^{INK4c}$. A similar contrasting pattern of $p18^{INK4c}$ and $p27^{kip1}$ expression is observed both in B cells activated in vivo and in various leukemic cells. Expression of $p18^{INK4c}$ was also detected in various Ig-secreting cell lines in which both maximum Ig secretion and specific $p18^{INK4c}$ expression were observed during the G1 phase. Our study shows that $p27^{kip1}$ and $p18^{INK4c}$ have different roles in B cell activation; $p27^{kip1}$ is involved in the control of cell cycle entry, and $p18^{INK4c}$ is involved in the subsequent early G1 arrest necessary for terminal B lymphocyte differentiation. *The Journal of Immunology*, 2000, 165: 4346–4352.

In most cell lineages, final differentiation is associated with loss of proliferation and cell cycle arrest in G1, which may or not be followed by a withdrawal from cell cycle. In response to Ag-specific and T-derived signals, human B lymphocytes proliferate before undergoing final differentiation into plasma secreting cells (1, 2). This differentiation takes place in nonproliferative cells and is therefore dependent on cell cycle regulation.

Cell cycle progression is under the control of a family of serine/threonine kinases named the cyclin-dependent kinases (cdks). Activation of cdk is regulated by their association with regulatory subunits (the cyclins), the expression of which is tightly regulated during the various steps of the cell cycle (3, 4). For instance, G1 progression is controlled by cdk4 and cdk6 associated with members of the cyclin D family. G1/S transition and S progression are controlled by cdk2 associated with cyclin E and cyclin A, respectively (5–7). One of their substrates is the retinoblastoma protein (pRb), which upon sequential phosphorylation by the cdk4/6/cyclin D and cdk2/cyclin E complexes releases the transcription factor E2F (8–14). E2F, in turn, activates genes required for the S phase (8). Although the activity of each complex is governed by the periodic accumulation of cyclins, additional levels of control include the phosphorylation and dephosphorylation of critical tyrosine and serine/threonine residues and the presence of specific cdk inhibitors (CKI) (3, 15). These inhibitors belong to two different families: one is the INK4 family, consisting of $p16^{INK4a}$ (p16), $p15^{INK4b}$ (p15), $p18^{INK4c}$ (p18), and $p19^{INK4d}$, and the other is the Cip/Kip family, consisting of $p21^{Cip1/Wa1/Sdi1}$ (p21), $p27^{kip1}$ (p27), and $p57^{kip2}$ (p57) (16–25). CKI belonging to the INK4 family interact preferentially with cdk4 and cdk6, preventing their association with the cyclins D, and therefore are specific for G1. The Cip/Kip proteins regulate cdk activity by forming ternary complexes with cdk and cyclins. They can regulate multiple cdk enzymes, including cdk4/6, throughout the cell cycle (15). Therefore, cdk4/6 are the only cdk to be regulated by both INK4 and Cip/Kip CKI, and this feature renders them pivotal in the control of both cell cycle entry and exit.

Studies of CKI-deficient mice suggest that despite common cdk targets shared by both families of CKI, these different inhibitors regulate various cell functions. For instance, disruption of p16 is associated with the development of spontaneous tumors at an early stage in various cell types, whereas p21-defective mice do not develop spontaneous tumors and are developmentally normal (20, 26–31). Both p18- and p27-deficient mice develop gigantism, disproportionally enlarged thymus and spleen, and putative tumorigenesis, which is regulated by p18 and p27 through two separate pathways controlling the function of pRb (31). In normal mice, p18 is widely expressed during embryogenesis and accumulates to high levels in a number of terminally differentiated tissues, whereas p27 is preferentially expressed in quiescent cells (12, 20, 32–37), suggesting that p18 and p27 regulate different aspects of cell activation.
We previously reported that cell cycle entry and G₁ progression of in vitro activated human B lymphocytes were dependent on both activation of the cdk6/cyclin D3 complex and down-regulation of p27 expression (12). Nevertheless, the mechanisms that control differentiation-coupled cell cycle arrest in normal human B cells are still poorly understood. In this paper we investigate the roles of different CKI during in vitro activation leading to final differentiation of normal B cell into Ig-secreting cells. We show that human B differentiation is dependent on G₁ cell cycle arrest, which is associated with inhibition of cyclin D3/cdk6-mediated pRb phosphorylation by p18. Thus, these data suggest that p27 and p18 are differentially involved in the control of the sequential cell cycle entry and exit observed during human B lymphocyte differentiation.

Materials and Methods

Reagents

Recombinant IL-10 was purchased from R&D Systems (Minneapolis, MN). Recombinant IL-2 was provided by Chiron Laboratory-France (Suresnes, France). Staphylococcus aureus Cowan 1 strain (SAC; pan-sorbin) was obtained from Calbiochem (La Jolla, CA), z-Val-Ala-n-Asp-fluoromethylketone was supplied by Bachem Biochemie SARL (Vosain le Bretonneux, France). Murine fibroblastic cells expressing both human CD40 ligand (CD40L) and CD32/FcγR1 (CD40L/CD32L) cells were gifts from Dr. Saeland (Scherings-Plough, Dardilly, France).

Cell preparations

Normal B cells were isolated from human tonsils as previously described (12). Briefly, single-cell suspensions were depleted of T cell by two cycles of negative selection with anti-methyl-isothiocyante-conjugated sheep anti-mouse Ig and were depleted of monocytes by adherence to plastic. B cells were then separated into density fractions on discontinuous Percoll gradients (Pharmacia, Uppsala, Sweden). Cells suspended below the 60% Percoll fraction (a homogeneous population of small cells, >97% IgD-CD38⁻) were considered to be resting cells, and cells collected at the 55% layer (large cells, >95% IgD⁺CD38⁺) were considered to be in vivo activated B cells. Leukemic B cells were isolated by negative selection after two cycles of T cell rosetting from blood obtained from patients with chronic lymphocytic leukemia-derived, IL-2-dependent Kitt 225 cell line.

Cell cycle analysis

Approximately 1.5 × 10⁶ cells were washed in PBS and resuspended in 1.5 ml of hypotonic fluorochrome solution (50 μg/ml propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100; Sigma). Samples were stored for 1 h at 4°C in the dark before flow cytometric analysis of propidium iodide fluorescence of individual nuclei using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Cell cycle compartments, including G₀/G₁, S, and G₂/M phases, and the percentage of the cells in the compartments were determined using the computer program CellFit DNA software with SFIT calculations (Becton Dickinson).

Western blotting

Cells were lysed by incubation in lysis buffer (50 mM Tris (pH 8), 150 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 50 mM NaF, 2 mM sodium vanadate, 1 μM ml aprotinin, 2 mM PMSF, 1 mM DTT, 10 μg/ml leupeptin, and 2 μg/ml pepstatin A) for 5 min on ice and 60 min. The lysate was centrifuged for 30 min at 13,000 × g at 4°C, and the supernatant was assayed for protein concentration (micro-bicinchoninic acid protein assay; Pierce, Rockford, IL). Cell lysate proteins (50 μg) were boiled for 5 min in 2× sample buffer and resolved on 10 or 15% SDS-polyacrylamide gels by electrophoresis. Proteins were electroblotted onto 0.45-μm pore size nitrocellulose filters, and the filters were blocked for 1 h at room temperature with 5% nonfat milk in PBS and 1% Tween-20. Filters were then incubated for 1 h with anti-cyclin D3 (2 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA), anti-cyclin E (2 μg/ml; C-198, Santa Cruz Biotechnology), anti-cdk2 (2 μg/ml; C-163, Santa Cruz Biotechnology), anti-cdk6 (2 μg/ml; C-21, Santa Cruz Biotechnology), anti-pRb (2 μg/ml; clone G3-245, Pharmingen, San Diego, CA), anti-p18 (2 μg/ml; Santa Cruz Biotechnology), or anti-actin (1 μg/ml). The blots were washed three times (10 min each time) with 0.1% Tween 20, pepsin (10 μg/ml) with pepsinase-labeled swine anti-rabbit or sheep anti-mouse Ig (1/5000; Amersham, Les Ulis, France). Blots were developed using an enhanced chemiluminescence detection system (ECL, Amersham). Films were exposed for 1–30 min.

Immunoprecipitation and kinase assay

Cell supernatants were incubated in microtiter plates coated with G₁ arrest. For retinoblastoma kinase assays, immune complexes were collected by centrifugation at 4°C and washed three times with buffer (50 mM HEPE (pH 7.2), 10 mM MgCl₂, 5 mM MnCl₂, 1 mM DTT, 50 mM NaF, 2 mM sodium vanadate, 1 μg/ml aprotinin, 2 mM PMSF, 1 mM DT, 10 μg/ml leupeptin, and 2 μg/ml pepstatin) and twice with kinase buffer (150 mM NaCl, 50 mM HEPES (pH 7.50), 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 2 mM sodium vanadate, 1 μg/ml aprotinin, 2 mM PMSF, 1 mM DT, 10 μg/ml leupeptin, and 2 μg/ml pepstatin) A. They were then suspended in a total reaction volume of 40 μl of kinase buffer containing 40 μg of [γ-³²P]ATP (3000 Ci/nmol; Amersham) and 0.5 μg of truncated retinoblastoma p56 pRb protein (QED Bioscience, San Diego, CA). After 1 h at 37°C, SDS-PAGE sample buffer was added, and the samples were heated to 100°C for 3 min on a 10% SDS-polyacrylamide gel. They were then transferred onto 0.45-μm pore size nitrocellulose filters, and autoradiographed for 16 h.

Results

Terminal B cell involvement is associated with G₁ arrest

To investigate the involvement of cell cycle regulatory molecules during human B lymphocyte differentiation, we first studied cell cycle progression during this process. For this, resting tonsillar B cells were activated in the presence of SAC (1/10,000), IL-2 (10 ng/ml), and IL-10 (100 ng/ml). Every day, progression of the activation and cell cycle progression during this process. For this, resting tonsillar B cells were activated in the presence of SAC (1/10,000), IL-2 (10 ng/ml), and IL-10 (100 ng/ml). Every day, progression of the activated B cells through the cell cycle was determined by flow cytometric analysis, and cell cycle differentiation was monitored as Ig production by measuring the concentration in the culture supernatant. Following activation, we observed a decrease in the percent-

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Regulation of p18^INK4^ expression during human B cell differentiation

Cell cycle progression is tightly regulated by specific cyclin/cdk complexes. We reported that the cyclin D3/cdk6 complex is the major regulator of the progression of human B cells to late G1. Indeed, this complex is directly responsible for pRb phosphorylation, which is a key marker of late G1 and the G1/S transition in B cells activated in vitro with mitogenic stimuli. We therefore investigated whether the G1 accumulation and expression of non-phosphorylated pRb observed in long-term activated B cells was due to down-regulation of either cyclin D3 or cdk6 expression. In contrast to resting B cells, cyclin D3 was present in 24-h-activated B cells (Fig. 2). The level of cyclin D3 expression remained constant until day 7. In parallel experiments we verified that among the cyclin D family, only cyclin D3 expression was up-regulated in our experimental conditions, and that cyclin D1 or D2 was not detectable in SAC/IL-2/IL-10-activated B cells (12) (data not shown). Because pRb phosphorylation during G1 was promoted by the cyclin D/cdk4 or cyclin D/cdk6 complexes, we next tested whether expression of cdk4 or cdk6 was down-regulated in activated cells re-entering G1. No significant regulation of cdk6 (Fig. 2) or cdk4 (data not shown) expression was observed during the time course of activation. Thus, G1 accumulation and the absence of pRb dephosphorylation observed in long-term activated B lymphocytes did not appear to be due to the reduced expression of either cyclin D3 or cdk6.

Study of p27 and p18 expression during B cell differentiation

The activity of cdk is also regulated by cdk inhibitors belonging to the INK4 and Cip/Kip families. We therefore investigated whether one or more of these inhibitors could be involved in the arrest of cell cycle progression observed during in vitro B cell differentiation. One member of the Cip/Kip family, p27, was strongly expressed in resting cells, and its expression decreased as long as these cells entered and progressed through the cell cycle. Its level of expression remained low even on days 6 and 7, when cells again accumulated in G1 (Fig. 3). None of the INK4 family inhibitors tested (p15, p16, p18, and p19) was present at a significant level in resting B cells (Fig. 3 and data not shown). Interestingly, upon activation with the combination of SAC/IL-2 and IL-10, an increase in p18 expression was observed. This up-regulation of p18 expression was apparent on day 6 and was almost maximal after 7 days of activation (Fig. 3). In contrast, p15, p16, and p19 expression levels were not modified (data not shown). Thus, these data show that p18 is highly expressed in activated cells that accumulate in G1 and suggest that p18 may be involved in and responsible for the absence of pRb phosphorylation in these stimulated cells.

Inhibition of pRb phosphorylation is associated with formation of cdk6/p18 complexes in vivo

Because the inhibitory activity of the INK4 inhibitors is specifically restricted to the cyclin D/cdk complexes controlling progression in G1, we investigated whether p18 is involved in the absence of pRb phosphorylation observed in G1-arrested cells. B cells were cultured for 48 h or 7 days with SAC, IL-2, and IL-10, and cell lysates were immunoprecipitated with either control rabbit Ig or anti-cdk6 Ab. The precipitates were analyzed for in vitro kinase activity using recombinant pRb as the substrate (Fig. 4). The cdk6 immune complex prepared on day 2 expressed a strong kinase activity compared with control Ig. In contrast, cdk6 immune complex isolated from cells activated for 7 days was not able to significantly phosphorylate pRb in vitro. These observations strongly support the hypothesis that p18 may be involved in the absence of pRb phosphorylation during G1 arrest.

Opposite expression of p27 and p18 during B cell differentiation

Human tonsillar B lymphocytes (10^6/ml) were activated for various times in the presence of SAC (1/10,000), IL-2 (10 ng/ml), and IL-10 (100 ng/ml). Cells were harvested at various times and tested for Ig content, cell cycle analysis was performed on cells of each sample, and the percentage of cells in G1 phase was determined as described in Materials and Methods. In parallel, cell lysates (50 μg) were tested for pRb by immunoblotting with anti-pRb Ab. The same membrane was then stripped and probed with anti-human actin Ab to assess the amount of protein loaded in each lane. Results are representative of at least three different experiments.
suggest that the in vivo pRb phosphorylation observed on day 2 was dependent on the kinase activity of the cyclin D3/cdk6 complex, whereas by day 7, cdk6 had lost its pRb phosphorylation activity. Because INK4 inhibitors abolish G1 cdk activity by disrupting the formation of cyclin D/cdk4/6 complexes, we investigated whether p18, present in day 8 activated B cells, could associate with cdk6. Indeed, p18 was found in cdk6 immune complexes prepared from 7-day-activated cells (Fig. 4). As expected, the cyclin D3/cdk6 complex was dissociated when p18 was associated with cdk6 on day 7 (Fig. 4), and the kinetics of the dissociation of the cyclin D3/cdk6 complex correlated with the kinetics of p18 expression (Figs. 3 and 5). In addition, we checked for the expression and activation of cdk2 which is involved in late G1, progression and, when associated with cyclin E, additionally phosphorylates pRb. As previously reported, no cdk2 was expressed in G0 or early G1 B lymphocytes (12). Cdk2 was preferentially expressed by day 3 of activation when most of the cells are cycling, and the level of cdk2 decreased to levels observed during the first 48 h of activation when cells were mostly in G1/G1 (Figs. 1 and 5). This expression of cdk2 was associated with activation, as indicated by the appearance of active cdk2 (visualized as a 33-kDa, faster migrating band corresponding to dephosphorylated cdk2) (19) and association with cyclin E (day 3 of Fig. 5). These data show that upon activation with the combination of SAC, IL-2, and IL-10, resting B cells enter the cell cycle, and their progression through G1 is associated with pRb expression and its phosphorylation by the cyclin D3/cdk6 complex. Accumulation of these cells in early G1, observed after 7 days of activation, is associated with up-regulation of p18 expression, which binds to cdk6, dissociates the cyclin D3/cdk6 complex, and inhibits its activity. Such inhibition of cdk6 results in the absence of pRb phosphorylation and therefore prevents activated B lymphocytes from re-entering the cell cycle.

**CD40-mediated B cell differentiation is also associated with up-regulation of p18 expression**

To validate our previous observations, we next investigated whether in vitro differentiation of human B lymphocytes following stimulation with CD40 was also associated with up-regulation of p18 expression (Fig. 6). Tonsillar resting B cells (10^5 cells/ml) were cultured for 7 days in the presence of irradiated murine CD40L/CD32L fibroblastic cells (5 × 10^3 cells/ml) and IL-10 (100 ng/ml). Culture supernatants were then tested for human Ig, and cells were harvested. A fraction of the cells was analyzed by Western blotting for cdk inhibitor expression, and cell proliferation was quantified by measuring radioactive thymidine uptake over 16 h by 10^5 cells (Fig. 6A) or by G1 cell cycle analysis, quantified by propidium iodide labeling and flow cytometric analysis of propidium iodide fluorescence of individual nuclei as well as cell counting (Fig. 6B). The remaining cells (10^7/ml) were cultured for one (day 14; Fig. 6A, 6 and 6B) or two (day 21; Fig. 6B) additional periods of 7 days with CD40L/CD32L cells and IL-10 in fresh medium. In these conditions these cells undergo several proliferative rounds, which can be detected by quantifying cells in G1. The number of cells capable of re-entering the cycle (as measured by

**FIGURE 4.** Inhibition of pRb phosphorylation is associated with formation of cdk6/p18 complexes in vivo. Human B cells (10^9/ml) were activated for 2 or 8 days in the presence of SAC (1/10,000), IL-2 (10 ng/ml), and IL-10 (100 ng/ml). Cell lysates were immunoprecipitated with either control rabbit serum (Ig) or anti-cdk6 Ab (cdk6), and kinase activity was assayed in vitro using recombinant pRb (γP32). After disappearance of radioactivity, the membrane was sequentially probed with anti-cdk6, anti-p18, and anti-cyclin D3 Abs (Western blot). Results are representative of two different experiments.

**FIGURE 5.** Kinetics of cdk2/cyclin E and cdk6/cyclin D3 complexes formation. Human tonsillar B lymphocytes (10^9/ml) were cultured for various times in the presence of SAC (1/10,000), IL-2 (10 ng/ml), and IL-10 (100 ng/ml). Every day, cell lysates were immunoprecipitated with either anti-cdk2 or anti-cdk6 Ab and subjected to Western analysis with anti-cdk2 or cyclin E Ab for anti-cdk2 immunoprecipitations and with cyclin D3 for anti-cdk6 immunoprecipitations, respectively. Results are representative of two different experiments.

**FIGURE 6.** CD40-mediated B cell differentiation is also associated with up-regulation of p18 expression. Human tonsillar B cells (10^9/ml) were cultured for 7, 14, or 21 days in the presence of irradiated CD32-CD40L cells and IL-10 (100 ng/ml). At the indicated time, supernatants were harvested and assayed for Ig content (A). Cells were counted, and the percentage of cells in G1 phase was determined as described in Materials and Methods and further cultured with freshly irradiated CD40L/CD32L cells and IL-10 in fresh medium (B). In parallel, DNA synthesis was quantified by measuring [3H]thymidine incorporation over 16 h for 10^5 cells from each group. Cell lysates (50 μg) were tested for p18 by immunoblotting with anti-p18 Ab. The same membrane was sequentially stripped and probed with anti-p27 and anti-human actin Ab (A). Results are representative of at least three different experiments.
determining the percentage of G₁ cells) decreased during the second period of culture (from days 7–14), and during the third period (days 14–21) cells remained in G₁, resulting in the absence of cell growth. In contrast, Ig secretion was detected on day 7, and Ig concentrations were much higher in supernatants from 7 to 14 days of culture, when cell growth was slow (Fig. 6), and remained stable on day 21 (data not shown). Once again, terminal differentiation into Ig-secreting cells was correlated with a large regulation of p18 expression. In contrast, p27, which was highly expressed in resting B cells, disappeared from activated cells. No major regulation of other cdk inhibitors was observed in these cells (data not shown).

**p27 and p18 expression in normal B cells activated in vivo and leukemic B cells**

To assess the particular expression of p18 and p27 during B cell activation and differentiation, we analyzed the expression levels of these two CKIs in both resting and in vivo activated tonsillar B cells isolated through Percoll gradient. As shown in Fig. 7, activated B cells (CD38⁺/IgD⁺; lane 2) expressed higher levels of p18 than did resting B cells (CD38⁻/IgD⁻; lane 1). In contrast, p27 expression was restricted to resting B cells. We next studied the expression of p27 and p18 in two different non-Ig-secreting chronic lymphocytic leukemia B cells (lanes 3 and 4) and in peripheral blood from a patient with Ig-secreting plasma cell leukemia (lane 5). We observed an opposite pattern of p18 and p27 expression in these different leukemia B cells. p27 was highly expressed in chronic lymphocytic leukemia B (B-CLL) cells and to a lesser extent in leukemic plasma cells, whereas p18 was very highly expressed in Ig-secreting leukemic cells and was absent from the two B-CLL cells.

**p27 and p18 expression in different cell lines**

Various cell lines, including Burkitt (Ramos, BL41, Daudi, Raji, and CAPA-2) and myeloma (U266 and LP1) cells, were examined for the expression of p18 and p27. All cells expressed significant amounts of p18. In contrast, p27 expression was either low or too low to be quantifiable in these cells (data not shown). All these cells, except for Daudi and Raji, which express, but do not secrete, Ig, are Ig-secreting cells. Thus, p18 expression correlated with the ability of B cell lines to produce Ig, although, as suggested by the expression of p18 in Daudi and Raji cells, its expression is not sufficient to ensure Ig secretion. In addition, p18 expression was observed in κ (BL41, Ramos, Daudi, Raji, and CAPA-2), γ (LP1), and ε (U266) Ig-expressing cells and thus was not correlated to a specific isotype. We next verified whether p18 expression was observed in these cells during all phases of the cell cycle or was restricted to the G₁ phase. For this, Ramos cells were arrested in G₁ by a 3-day culture in the absence of FCS (but in the presence of the broad caspase inhibitor z-Val-Ala-D,L-Asp-fluoromethylketoene to prevent apoptotic cell death). G₁ arrest of Ramos cells (assessed by both cell cycle analysis and pRb dephosphorylation) was associated with up-regulated p18 expression (Fig. 8, compare lane 2 to control nonsynchronized cells in lane 1). Concomitantly, the relative amounts of Ig in the supernatant also increased, suggesting that maximum Ig secretion was correlated to G₁ cell cycle arrest and expression of p18. Furthermore, following induction of cell cycle progression by the addition of FCS, p18 expression and Ig secretion were reduced in Ramos cells (Fig. 8, lanes 3 and 4). No significant modulation of p27 was observed in cycling and G₁-arrested Ramos cells, whereas p27, but not p18, was highly up-regulated in the IL-2-dependent Kitt 225 T cell line arrested in G₁ by IL-2 deprivation (Fig. 8, lanes 5 and 6).

**Discussion**

In this paper we investigate the interplay between the regulation of cell cycle progression and the final differentiation process in human B lymphocytes. Using different stimulatory combinations, we observed that Ig secretion by normal tonsillar B cells occurs when cells stop progressing through the cell cycle and accumulate in G₁.

The number of cell cycle divisions is variable according to the nature of the stimulus. For instance, after stimulation with the combination of the B cell mitogen SAC, IL-2, and IL-10, accumulation of activated cells in G₁ and subsequent Ig secretion take place after a single round of proliferation, whereas G₁ arrest and Ig secretion observed after activation with CD40 and IL-10 occur after several rounds of proliferation, leading to quantifiable cell growth (Fig. 6B) (40, 41). Hashbold et al. reported that after CD40 and IL-4 stimulation of murine B cells, IgG secretion was observed after three rounds of proliferation, whereas IgE production was only evidenced after five proliferative rounds (42). This strongly suggests that, at least in vitro, isotype specificity of secreted Ig is related to the number of proliferative rounds, consistent with the assumption that isotype switching preferentially takes place during S progression (43, 44). Nevertheless, Ig secretion was always detected in our experimental conditions when cells were either arrested in early G₁ or leaving the cell cycle. The behavior of pRb may be a marker of this cell cycle exit. Thus, we observed that the

**FIGURE 7.** High p18 expression in in vivo activated normal B cells and plasma cell leukemia cells. Resting and in vivo activated B cells were purified by Percoll fractionation as described in Materials and Methods. p18 and p27 expression was analyzed in resting (lane 1) or in vivo activated normal B cells (lane 2) and in chronic lymphocytic leukemia B cells (lanes 3 and 4) and leukemic plasma cells (lane 5). Cell lysates (50 μg) were tested for p27 by immunoblotting with anti-p27 Ab. The same membrane was sequentially stripped and probed with anti-p18 and anti-human actin Ab. Results are representative of two different experiments.

**FIGURE 8.** G₁ arrest of Ramos cells is associated with up-regulation of p18 expression. Ramos cells were cultured for 3 days in the presence (lane 1) or the absence (lane 2) of FCS. G₁-arrested Ramos cells (lane 3) were cultured for an additional 3 days in the presence of FCS (lane 4). IL-2–dependent Kitt 225 T cells were cultured in the presence (lane 5) or the absence (lane 6) of IL-2 (10 ng/ml), such that they were synchronized or not synchronized in G₁, respectively. p18 was revealed by immunoblotting with anti-p18 Ab in cell lysates (50 μg). The same membrane was sequentially stripped and probed with anti-p27, anti-pRb, and anti-human actin Abs. Culture supernatants were harvested, and cells were enumerated. Ig content is expressed as nanograms of total Ig per 10⁶ cells. Cell cycle analysis was performed on cells of each sample, and the percentage of cells in the G₁ phase was determined as described in Materials and Methods. Results are representative of at least two different experiments.
G1 accumulation observed from day 5 was associated with the dephosphorylation of pRb, which is the pRb pattern in early G1 (Fig. 1). Moreover, terminal differentiation, evidenced by Ig secretion observed on days 7 and 8, was associated with a significant decrease in the total pRb level, which was not compensated by an increase in the amounts of other members of the pRb family such as p130 or p107. This absence or lower pRb expression was also observed in resting B cells that had not yet entered the cell cycle and is probably a property of B lymphocytes that are not cycling (45). Ig secretion also takes place during the G1 phase in cycling B cell lines, because Ig secretion by Ramos cells was significantly increased when they were arrested by FCS deprivation in early G1.

G1 progression is regulated by the kinases cdk4 and cdk6, which are able, after association with cyclin D, to phosphorylate different substrates, including pRb. Such phosphorylation of pRb is correlated with the passage through the G1 restriction point (R1). Additional phosphorylations of pRb by the cdk2/cyclin E complex are necessary for the G1 to S transition (8–10, 14). In human B lymphocytes, cell cycle entry, triggered by mitogenic activation by IL-4 in combination with either anti-CD40 or anti-CD40 Ab, is dependent on expression of cyclin D3 and, to a lesser degree, of cyclin D2. Cyclin D3 associates preferentially with cdk6, and the cyclin D3/cdk6 complex is responsible for the pRb phosphorylation observed both in vivo and in vitro (12). A similar pattern was observed when B cells were stimulated with IL-10 in combination with IL-2 and SAC. Cyclin D3, which is not expressed in resting B cells, was rapidly induced in 24-h-activated cells and was associated with cdk6 to form a cyclin D3/cdk6 complex responsible for pRb phosphorylation (Figs. 2 and 4). Nevertheless, although total amounts of cdk6 and cyclin D3 were not significantly modified, cdk6 was not able to phosphorylate pRb on day 7 of culture when these cells were arrested in early G1 (Figs. 1, 2, and 4). In contrast, expression levels of cdk2 and cyclin E are highly regulated during cell cycle, and neither early (0–48 h) nor late (6–7 days) activated cells express cyclin E and cdk2. This observation is in accordance with the hypothesis that cell cycle arrest observed in our experimental conditions occurs in early G1. In addition to regulatory phosphorylations, the activity of cdk6 is closely dependent on the CKI proteins (3, 15). This mechanism, involving CKI activity, is probably responsible for the cdk6 inhibition observed on day 7 of culture, because 1) in contrast to p27, the expression of which decreases upon activation, p18 expression was strongly up-regulated in G1-arrested cells (day 7); and 2) the inactive cdk6 isolated from these cells was associated with p18 (Figs. 3 and 4). A similar up-regulation of p18 expression was observed in both B cells activated in vitro with IL-10 and anti-CD40 Ab or B cells activated in vivo (Figs. 6 and 7). In addition, high p18 expression was observed in cells from a patient with plasma cell leukemia, whereas no p18 was present in two different nonsecreting B-CLL cell lines (Fig. 7). These observations show that p18 is preferentially expressed in activated B cells engaged in final differentiation. This conclusion is in accordance with the previous observations that p18 is also up-regulated in EBV-positive CESS cells upon stimulation with IL-6 (which leads to cell cycle arrest and enhanced Ig secretion) as well as during myogenesis (46, 47).

Beside their role in regulating cdk, CKI may also be involved in other cellular functions. For instance, different groups reported that p21 can exhibit anti-caspase properties (48). Therefore, it would be useful to determine whether in addition to its involvement in cell cycle regulation, p18 is directly involved in the differentiation process itself. Additional experiments, including transfection of different B cell types with p18, are in progress to determine whether p18 may directly regulate the process of Ig heavy chain production. Nevertheless, the augmented Ig production observed in G1-arrested Ramos cells was associated with a specific increase in p18 with no apparent regulation of other CKI, underlining the particular pattern of p18 expression in differentiating cells already observed in normal B cells.

Interestingly, p18 expression and p27 expression appear to be mutually exclusive in the cell types we studied. Thus, normal B cells activated either in vitro or in vivo expressed large amounts of p18 and almost no p27, whereas resting B cells expressed p27 but not p18. A similar dichotomy was observed between B-CLL cells and leukemic plasma cells as well as in the different cell lines analyzed. The significance of this dissociation is not yet clear. A possible explanation for the differential pattern of p18 and p27 expression may be the specificities of their inhibitory activities during the cell cycle. Indeed, p18 is restricted to G1, cdk and associates preferentially with cdk6, which is the principal cdk controlling G1 progression in human B cells (12, 20). p27 can interact with various cdk controlling the different cell cycle steps. Thus, p27 overexpression would be more potent in preventing resting B cells from entering the cell cycle. In contrast, as B cell differentiation requires a specific and mandatory stop in early G1, such arrest may be more specifically controlled by the specific inhibitions of cyclin D3/cdk6-mediated pRb phosphorylation by p18. The differential involvement of p18 and p27 during the control of B cell activation can also be observed in p18- and p27-deficient mice. Indeed, although both deficient mice exhibited disproportionately enlarged and hyperplastic spleens, only B cells from p18-deficient mice showed a higher proliferative rate upon in vitro mitogenic activation with CD40L (31). Alternatively, as terminal differentiation is preferentially associated with apoptosis (see discussion above), the different patterns of p18 and p27 expression observed in B cells could be related to differential involvement of these two CKI in the control of the apoptotic process. Indeed, high expression of p18 was very often observed in cells highly sensitive to apoptosis, whereas expression of p27 was observed in cells, such as resting cells or the Kitt 225 T cells, more resistant to spontaneous in vitro apoptosis (data not shown). Additional experiments are necessary to elucidate the exact roles of p18 and p27 in the regulation of the sensitivity of normal B cells to apoptosis.

In conclusion, our data show that the two CKI, p18 and p27, are involved in different steps of B cell activation. Whereas p27 is associated with control of cell cycle entry of resting cells, p18 (through inhibition of cyclin D3/cdk6-mediated pRb phosphorylation) is more directly involved in induction of the G1 cell cycle arrest necessary for terminal B lymphocyte differentiation.

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References


