

# Induction of Cell Cycle Regulatory Proteins in Anti-Immunoglobulin-stimulated Mature B Lymphocytes

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## Summary

Progression through the cell cycle is a tightly controlled process that integrates signals generated at the plasma membrane with the proteins that form the cell cycle machinery. The current study chronicles the induction of cyclins, cyclin-dependent kinases (cdk), and cdk inhibitors in low density primary mouse B lymphocytes after anti-immunoglobulin plus interleukin 4 (IgM + IL-4) stimulation. In this system, >85% of cells remain in the G0/G1 phase of cell cycle for an initial 24-h period, followed by entry of up to 50% of the cells into S phase, commencing around 30 h and peaking at 48 h. Extensive time course analyses of these anti-IgM + IL-4-stimulated B cells revealed that the G1-associated D-type cyclins D2 and D3 were induced by 3 h after stimulation, and that cyclins E, A, and B were subsequently induced sequentially, beginning at mid-G1, G1/S transition, and S phase, respectively. The G1-associated cyclin D1 was not expressed at any stage of the anti-Ig + IL-4-induced B cell cycle. cdk2, cdk4, and cdk6 were induced during G1, whereas cell division cycle-2 (cdc2) was induced concomitantly with S phase. Irrespective of their expression, the kinases cdk2 and cdc2 were only active from S phase onwards, suggesting that productive cyclin/kinase complex formation did not occur until that time. Cell cycle inhibitors p21 and p19 were induced by anti-Ig + IL-4, peaking in expression at mid-G1 and S phase, respectively. Stimulation of low density B cells with anti-Ig + IL-4 caused rapid down regulation of the p27 inhibitor, however this protein was reexpressed at 54–96 h after stimulation. In contrast, B cells stimulated with anti-CD40, a stimulus which induces long-term B cell proliferation, permanently down regulated p27. These findings are consistent with the concept that p27 reexpression contributes to the G1 arrest that follows antigen receptor crosslinking. Low density B cells cultured in the viability-enhancing cytokine IL-4 alone also showed induction of D2 and D3 cyclin expression. However, the D2 expression was transient, and the D3 expression was substantially lower than that observed in B cells induced to proliferate by anti-Ig + IL-4. This partial induction of D2 and D3 expression may explain IL-4's ability to promote B cell entry into G1 but not S phase of cell cycle, and furthermore, its ability to truncate G1 progression when B cells are subsequently stimulated with anti-Ig.

**A**ntibody production by B lymphocytes is a fundamental effector mechanism of the immune system. This response is critically dependent on the extent of B cell replication that follows antigen stimulation, since proliferation of activated B cells allows extensive expansion of initially small numbers of antigen-specific B cell precursors (1 and for a review see reference 2). The regulation of antigen-initiated B cell replication has been the subject of intense investigation for many years (for reviews see references 2–4). Such studies have included experiments that show that engagement of the antigen receptor using appropriate anti-Ig antibodies causes essentially all resting splenic mouse B cells to transit from G0 to G1 in the cell cycle, with 50–60% of these cells completing the S, G2, and M phases of cell cycle

in a 40–48-h culture period (5, 6). Most of these B cells appear to G1 arrest after this initial round of replication, although a small subset may go through a second round of replication (7, 8). These cell cycle events are accompanied by an array of biochemical reactions (9–11) including tyrosine phosphorylation (12, 13), activation of protein kinase C (14, 15), and calcium mobilization (16), all of which occur rapidly after antigen cross-linking.

The proliferative response of B cells stimulated with anti-Ig antibodies can be enhanced by coculture of the cells with the T cell-derived cytokine IL-4 (17). IL-4 is not an essential activation signal in this system, since the proliferation of B cells stimulated with supersaturating concentrations of anti-Ig is unaffected by anti-IL-4 antibodies (7).

Similarly, B cells from IL-4 knockout mice (18) produce a normal proliferative response to *in vitro* stimulation with supersaturating concentrations of anti-Ig (our unpublished data). While not an essential cofactor, IL-4 enhances anti-Ig stimulation of B cells by increasing the sensitivity of B cells to the anti-Ig signal (19), by increasing the proportion of B cells that enter S phase after anti-Ig stimulation (8, 19), and by providing an anti-apoptotic signal (20) that accounts for the observed increase in B cell viability (19, 21, 22). IL-4 also enables a large proportion of B cells to complete the second and third rounds of the cell cycle after stimulation through the antigen receptor (8). In addition to its ability to enhance anti-Ig-induced B cell proliferation and maintain B cell viability, IL-4 has a number of other direct effects on resting B cells. Specifically, IL-4 causes an increase in B cell size (21, 23), and upregulates cell surface expression of class II MHC antigens (23, 24), Fc $\epsilon$ R (22, 25, 26), and IL-4 receptors (27). Furthermore, B cells cultured in IL-4 for 24 h before incubation with anti-Ig will enter S phase 12 h earlier than non-IL-4-exposed control cultures (7, 28, 29). IL-4 transmits signals to B cells and other cells via binding to an IL-4-binding protein (30, 31) that subsequently causes activation of an associated signal-transducing protein known as the common  $\gamma$  chain (32, 33). The latter event initiates a chain of biochemical reactions, including activation of the Jak3 tyrosine kinase (34, 35), which in turn phosphorylates Stat6 (36).

Whereas seemingly much is known about biochemical reactions that become triggered in the first minutes-to-hours after a B lymphocyte is stimulated with anti-Ig + IL-4, little is known of the ensuing intracellular events that comprise the remainder of the 40–48-h cell cycle period. In particular, the early biochemical events described above are yet to be integrated with the family of cyclins, kinases, and inhibitors that comprise the cell cycle machinery responsible for regulating cellular replication. The cyclins are primary regulators of progression through the cell cycle, serving as regulatory subunits for a family of serine/threonine kinases known as the cyclin-dependent kinases or cdk<sup>1</sup> (for reviews see references 37, 38). Cyclins D and E, with their associated kinase partners, control cell cycle checkpoints that occur early and late in G1, respectively (39–41). Cyclin A/cdk complexes are active initially at the onset of S phase and throughout G2 (42, 43). The induction of cyclin B during S phase, and subsequent accumulation of cyclin B/cell division cycle-2 (cdc2) complexes at G2, control entry into mitosis (for reviews see references 44, 45). Thus, the sequential progression through the cell cycle is ultimately controlled by the temporal induction and activation of a series of different cyclin/cdk kinases. The enzymatic activity of cyclin/cdk complexes can be modified by association with a variety of inhibitory molecules by mechanisms that are not well understood (for a review see reference 46). The inhibitors can be divided into two groups, those

that bind multiple cyclin/cdk complexes (e.g., p27 and p21) and thus are broad regulators of cell cycle, and those that have a preference for G1-associated D cyclin/cdk complexes (INK4 family, e.g., p19). These inhibitory molecules, by virtue of their ability to modulate kinase activity, are potent cell cycle regulators.

In this study, we explore the induction of cell cycle regulatory proteins in normal murine B lymphocytes that are induced to proliferate by cross-linking their antigen receptors in the presence of the viability-enhancing cytokine IL-4. Our data indicate that proliferating mouse B lymphocytes do not express cyclin D1, but otherwise exhibit a typical cell cycle “clock” of cyclins, cdk, and inhibitors.

## Materials and Methods

**Mice.** 8–10-wk-old BALB/c mice were obtained from Simonson (Gilroy, CA) and were used as a source of low density splenic B cells throughout this study.

**Reagents.** Antibodies used in this study were monoclonal rat anti-mouse cyclins D3 and D2, mouse anti-mouse cyclin D1, and rabbit anti-mouse cyclins E, A, and B, p27, p21, p18, cdk2, cdk4, and cdk6; these reagents were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse anti-mouse cdc-2 and mouse anti-human-retinoblastoma (Rb) antibodies used for Western blotting were purchased from PharMingen (San Diego, CA). Horseradish peroxidase-conjugated anti-rat, rabbit, and mouse secondary reagents were purchased from Amersham Corp. (Arlington Heights, IL). Additional antibodies against cdk4 and p19 were kindly provided by Dr. Charles Sherr (St. Jude Children's Research Hospital, Memphis, TN). Polyclonal rabbit anti-mouse p27 was kindly provided by Drs. Hideo Toyoshima and Tony Hunter (The Salk Institute, San Diego, CA). The anti-cdc2 antibody used for immunoprecipitations was purchased from GIBCO BRL (Gaithersburg, MD). The rabbit anti-mouse cdk2 was prepared as described previously (47). The substrate for the kinase assay, histone H1, was purchased from Boehringer Mannheim Corp. (Indianapolis, IN). The affinity-purified F(ab')<sub>2</sub> goat anti-mouse IgM used to stimulate B cells was purchased from Cappel (Durham, NC), and the recombinant mouse IL-4 was produced in house at DNAX Research Institute. The rat anti-mouse monoclonal anti-CD40 antibody was described previously (48). The HT-2 proliferation bioassay used to quantitate IL-4 activity was performed as described elsewhere (48), with a unit of activity defined as the amount of cytokine producing 50% maximal response.

**Purification of Low Density B Cells.** To isolate splenic B cells, single cell suspensions were prepared from spleens of unprimed mice as described in detail elsewhere (19, 48). Briefly, red blood cells were lysed using red blood cell lysis buffer (Sigma Chemical Co., St. Louis, MO) according to the manufacturer's instructions. The cells were then stained using anti-Thy-1 (NEN Research Products, Wilmington, DE) and anti-CD4 (RL172) for 20 min on ice, followed by incubation in rabbit complement (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) at 37°C for 45 min. Low density B cells were then isolated by density gradient centrifugation using a discontinuous gradient composed of 85, 65, and 50% Percoll (Sigma Chemical Co.) and centrifuging at 1,500 g for 20 min with the brake off. Cells between 50 and 65% Percoll, corresponding to a density range of 1.062–1.0819 g/ml, were collected for experiments. These cells were >90% Ig bearing and

<sup>1</sup>Abbreviations used in this paper: cdc2, cell division cycle; cdk, cyclin-dependent kinase; Rb, retinoblastoma.

represented ~60% of the total B lymphocytes recovered after density centrifugation, containing most of the large, low density B lymphocytes.

**In Vitro Stimulations and Whole Cell Lysate Preparation.** 50-ml cultures were set up at  $10^6$  B cells/ml in 75 cm<sup>2</sup> flasks (Becton Dickinson & Co., Lincoln Park, NJ) and stimulated with 10  $\mu$ g/ml F(ab')<sub>2</sub> anti-IgM with or without 100 U/ml IL-4 or with 100 U/ml IL-4 alone for the initial time course experiments. Additional cultures were set up using anti-IgM (25  $\mu$ g/ml) or anti-CD40 (25  $\mu$ g/ml) in the absence of IL-4. All stimulations were carried out in supplemented RPMI (designated cRPMI) containing 10% FCS (JR Scientific, Woodland, CA),  $5 \times 10^{-5}$  M 2-ME (Polysciences, Inc., Warrington, PA), 2 mM glutamate (JR Scientific), 10 mM HEPES buffer (Irvine Scientific, Santa Ana, CA), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (Irvine Scientific). Cells were stimulated for the times indicated. After stimulation, cells were recovered, washed three times in cold PBS, divided into three pellets, and stored at -80C for later use in Western blotting and in vitro kinase assays. When all the time points were collected, one pellet from each time point was lysed in NP-40 lysis buffer (1% NP-40, 250 mM NaCl, 1 mM HEPES, pH 7.5, and 1 mM dithiothreitol [DTT; United States Biochemical Corp., Cleveland, OH]) with protease inhibitors added (final concentration, 5  $\mu$ g/ml aprotinin [Sigma Chemical Co.], 125  $\mu$ g/ml Pefabloc [Boehringer Mannheim Corp.], 5  $\mu$ g/ml leupeptin [Sigma Chemical Co.], and 5  $\mu$ g/ml pepstatin [Sigma Chemical Co.]). Protein concentrations were calculated using a Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's procedures. An equal volume of 2X SDS sample buffer (Novex, San Diego, CA) with 2-ME was then added to the total cell lysates. 125  $\mu$ g of lysate was run per time point on 12% Novex reducing gels according to the manufacturer's suggested protocol (6% gels were used to detect the Rb protein), and transferred onto Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) at 30 V overnight at 4°C. P3X or NIH3T3 cells were used as positive controls on all gels.

**In Vitro Kinase Assay.** cdk2- and cdc2-dependent kinase assays were performed using the histone substrate according to procedures described elsewhere (49). Briefly, frozen pellets were lysed in 1 ml 0.1% NP-40 lysis buffer containing 50 mM HEPES, pH 7.0, and 250 mM NaCl in the presence of the above protease inhibitors. Protein concentration was calculated as described above, and 200  $\mu$ g of total protein was used for each kinase assay. Briefly, lysates were precleared with normal rabbit serum followed by Zysorbin (Zymed Laboratories Inc., South San Francisco, CA). 1  $\mu$ g of anti-cdk2, anti-cdc2, or normal rabbit serum was added to the lysate and incubated at 4°C for 4 h with rocking. Protein G beads (Pharmacia, Milwaukee, WI) were added and the lysates were rotated in the cold for another hour. Immunoprecipitates were washed four times in lysis buffer followed by two washes in kinase buffer (50 mM Tris, pH 7.4, 10 mM MgCl<sub>2</sub>, and 1 mM DTT). The kinase reaction was carried out in a 50- $\mu$ l volume containing 2.5  $\mu$ g histone H1 and 5  $\mu$ Ci  $\gamma$ -[<sup>32</sup>P]ATP (Amersham Corp.) per reaction. Reactions proceeded for 30 min at room temperature with rocking. Samples were run on 12% Novex precast gels, fixed for 10 min in 50% methanol and 10% acetic acid, dried, and exposed to film for 3 h at room temperature.

**Cell Cycle Analysis.** Cell cycle analyses were performed using a modification of the method described by Crissman et al. (50). Aliquots of stimulated cells were taken at times indicated and washed twice in PBS. Single cell suspensions were added dropwise to ice cold 100% ETOH while vortexing, and stored at 4°C overnight. Cells were then centrifuged and resuspended in pro-

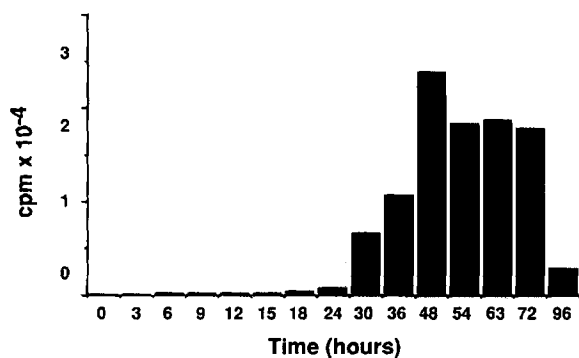
pidium iodide/RNase A solution (10  $\mu$ g/ml [Sigma Chemical Co.] and 250  $\mu$ g/ml [United States Biochemical Corp.], respectively), followed by incubation at 37°C for 30 min. Viable cells were analyzed by CellFIT (Becton Dickinson Immunocytometry Systems, San Jose, CA) software and the proportion of cells in each phase of cell cycle was calculated.

**[<sup>3</sup>H]Thymidine Incorporation.** 200  $\mu$ l aliquots ( $2 \times 10^5$  total cells) of stimulated cells was placed in 96-well flat bottom plates at times indicated and pulsed for 45 min with 1  $\mu$ Ci [<sup>3</sup>H]thymidine (Amersham Corp.). Cells were harvested and incorporated counts per minute were counted using a PHD cell harvester (Cambridge Technology Corp., Cambridge, MA).

## Results

**Large B Cells Enter S phase at ~30 h after Stimulation with Anti-IgM + IL-4.** To study the induction of cell cycle regulatory proteins in anti-IgM-stimulated B lymphocytes, we used a population of B lymphocytes that was purified by negative selection from spleens of unprimed BALB/c mice. The resultant B cell population was enriched for large, low density B cells and represented ~60% of the total B cells recovered after density centrifugation. This population was highly purified for B lymphocytes, since 90% of cells coexpressed B220 and IgM membrane proteins. These enriched large B cells were selected for our studies rather than highly purified small dense resting B cells since the low yield of the latter cells, together with their marked susceptibility to rapid death in vitro, severely restricted biochemical analyses. Comparative studies indicated that the enriched large B cells entered S phase of cell cycle ~6 h earlier than highly purified small dense B cells under the experimental conditions employed, but otherwise exhibited essentially identical growth characteristics (data not shown). The cell cycle status of the starting population of enriched large B cells was evaluated by propidium iodine staining and FACS<sup>®</sup> analysis (Becton Dickinson & Co.), as described in detail elsewhere (19). These analyses indicated that in several independent preparations of low density B cells, the majority of cells (i.e., 85-96%) was in the G0/G1 phase of cell cycle (data not shown).

Enriched large B cells were cultured at  $10^6$ /ml and stimulated with 10  $\mu$ g/ml anti-IgM plus 100 U/ml IL-4. Cell aliquots were collected at 0, 3, 6, 9, 12, 15, 18, 24, 30, 36, 48, 54, 62, 76, and 96 h after stimulation for analysis. Cell cycle status of the cells was monitored by propidium iodine staining and FACS<sup>®</sup> analysis, and DNA synthesis was measured by [<sup>3</sup>H]thymidine incorporation. Both approaches revealed that, in all experiments evaluated, a large proportion of cells commenced entry into S phase of cell cycle ~30 h after stimulation (a typical experiment is shown in Fig. 1). Peak [<sup>3</sup>H]thymidine incorporation occurred at 48 h after stimulation when ~60% of the cells were found in the S, G2, or M phase of cell cycle. As previously described (8), a large proportion of low density B cells progress to the second and even third rounds of the cell cycle when stimulated with anti-IgM + IL-4 resulting in the high [<sup>3</sup>H]thymidine incorporation detected at 54-72-h time points.



G <sub>0</sub> /G <sub>1</sub>	85	84	87	90	84	88	88	87	75	68	38	47	49	63	85
S	3	3	1	2	2	2	2	2	14	21	48	33	35	21	6
G <sub>2</sub> /M	12	14	12	9	15	11	10	10	12	13	14	20	16	15	10

**Figure 1.** Cell cycle status of low density primary mouse B lymphocytes after stimulation with anti-IgM + IL-4.  $10^6$  cells/ml were stimulated with 10  $\mu$ g/ml anti-IgM plus 100 U/ml IL-4. 200  $\mu$ l aliquots were taken at 0, 3, 6, 9, 12, 15, 18, 24, 30, 36, 48, 54, 62, 74, and 96 h after stimulation and pulsed with 10  $\mu$ Ci [<sup>3</sup>H]thymidine in 96-well flat bottom plates for 45 min (top). To perform cell cycle analysis,  $10^6$  cells were removed at times indicated, stained with propidium iodide, and analyzed by flow cytometry. The proportion of cells in each phase of cell cycle was determined using CellFIT software (bottom). Similar results were obtained in a second independent experiment.

This analysis of the anti-Ig-induced B cell cycle is highly consistent with similar studies described previously by DeFranco, A. et al. (5, 6).

**Induction of G<sub>1</sub>-associated Cyclins and cdk in B Cells Stimulated with Anti-IgM + IL-4.** In other cell systems, the D-type cyclins and their kinase partners cdk4 and cdk6 are the earliest complexes induced as cells leave quiescence, and are therefore believed to play a critical role in exit from G<sub>0</sub> (for a review see reference 37). To evaluate this phase of cell cycle in primary B lineage cells, purified large B cells that had been stimulated in vitro for different time periods with anti-IgM + IL-4 were lysed, and their intracellular proteins analyzed for expression of G<sub>1</sub>-associated cell cycle regulatory proteins by Western blot analysis. Cyclins D2 and D3 were usually not detected in unstimulated B cells; however, they were induced rapidly (i.e., by 3 h) and synchronously after stimulation, and thereafter exhibited a protracted appearance throughout the entire B cell cycle (Fig. 2 A). Cyclin D2 migrated as a single species throughout the time course, reaching peak levels by 6 h and remaining constant throughout the time course until 96 h. In contrast, activated B cells expressed multiple forms of cyclin D3 at different stages of cell cycle (Fig. 2 A). The functional significance of this heterogeneity is yet to be established.

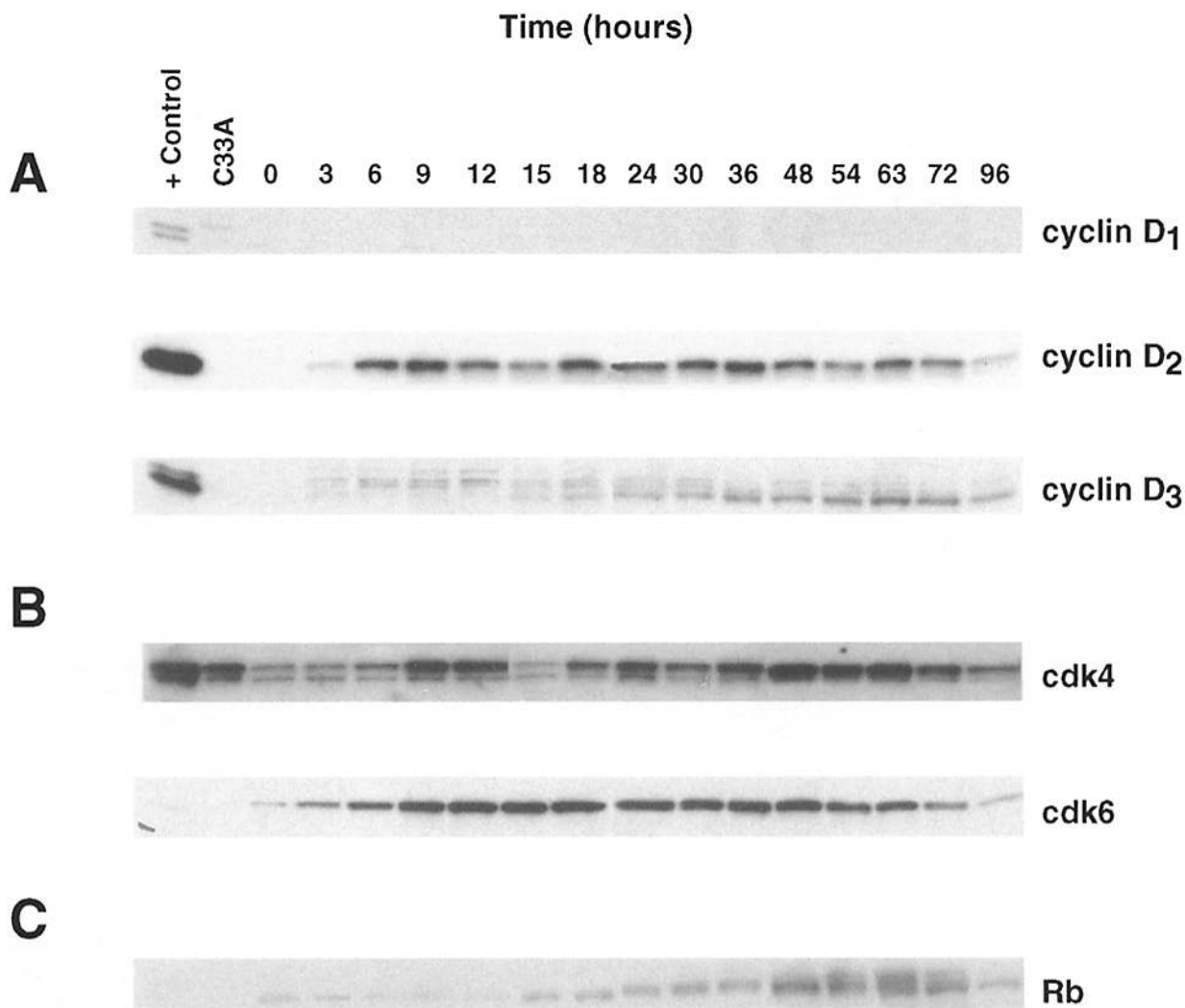
In contrast to cyclins D2 and D3, cyclin D1 was not detected at any time point after stimulation of B cells with anti-IgM + IL-4 (Fig. 2 A). Control experiments revealed that the sensitivity of cyclin D1 detection on our experiments was  $\sim$ 3 ng (our unpublished data). Our finding that cyclin D1 was not induced in activated B lymphocytes was supported by separate experiments showing that none of 13

cell lines derived from murine B cell malignancies expressed cyclin D1 (not shown). These data together suggest that proliferating mature murine B cells do not utilize cyclin D1 for cell cycle progression.

The preferred kinase partners for the D-type cyclins are cdk4 and cdk6. When B cells stimulated with anti-IgM + IL-4 were analyzed for cdk expression, the data showed that both cdk4 and cdk6 were upregulated or induced early in G<sub>1</sub>, at 9 and 3 h, respectively (Fig. 2 B). In other cell systems, the binding of D-type cyclins to its kinase partners results in the appearance of an active kinase that is believed to phosphorylate Rb, allowing release of bound transcription factors such as E2F that are required for the entry of cells into S phase (51–55). Since attempts to specifically assay this kinase activity in our experiments were unsuccessful technically, we employed an alternative approach of monitoring the appearance of endogenous phosphorylated Rb after B cell activation. Our experiments indicated that the first detectable in vivo phosphorylation of the endogenous Rb after anti-IgM + IL-4 stimulation of B cells occurs at 18 h (Fig. 2 C), some 15 h after the appearance of the D-type cyclins and cdk6, and 9 h after the appearance of cdk4 (Fig. 2 A). By 24 h, there is a slight upward mobility shift in the Rb gene product, and multiple phosphorylated forms can be seen at later time points (Fig. 2 C). At 96 h after stimulation, a time when the cells have exited the cell cycle in this anti-Ig stimulation system (see Fig. 1), the Rb gene product returns to a hypophosphorylated form (Fig. 2 C).

**Induction of G<sub>1</sub>/S-, S-, and G<sub>2</sub>/M-associated cyclins, cdk, and Associated Kinase Activity in B Cells Stimulated with Anti-Ig + IL-4.** In other cell systems, the G<sub>1</sub>/S interphase of cell cycle, and subsequent progression to G<sub>2</sub> and M phases, are regulated by cyclins E, A, and B and by their preferred kinase partners cdk2 and cdc2 (for reviews see references in 37, 38). The expression of these cell cycle regulatory proteins in normal B cells stimulated with anti-IgM + IL-4 was evaluated by Western blot analysis of activated B cell lysates. Cyclin E, a regulator that is required for cells to transit the G<sub>1</sub>/S interphase (39, 41), was first induced at  $\sim$ 9 h after stimulation (Fig. 3 A), i.e., some 6 h after D2 and D3 induction (Fig. 2 A). Like the D-type cyclins, cyclin E is also detected throughout the remaining 96-h response period. The protracted appearance of cyclin E in this time course is likely the consequence of a large proportion of the cells entering subsequent rounds of the cell cycle. Cyclin A, a regulator that is intimately associated with DNA replication (for a review see reference 56), was first detected at 24 h (Fig. 3 A), just before the entry of cells into S phase (see Fig. 1). Cyclin B, which is normally associated with G<sub>2</sub>/M, was first detected after cyclin A induction, at about 30 h after stimulation (Fig. 3 A). Expression of cdk2, the preferred kinase partner for cyclins E and A, is upregulated in G<sub>1</sub> and reaches maximal expression during S phase (Fig. 3 B, top). Expression of cdc2, the kinase partner for cyclin B and to a lesser extent cyclin A, is induced at  $\sim$ 30 h (Fig. 3 C, top), concomitantly with the entry of cells into S phase (see Fig. 1).

In the case of cdk2 and cdc2, effective cyclin/kinase

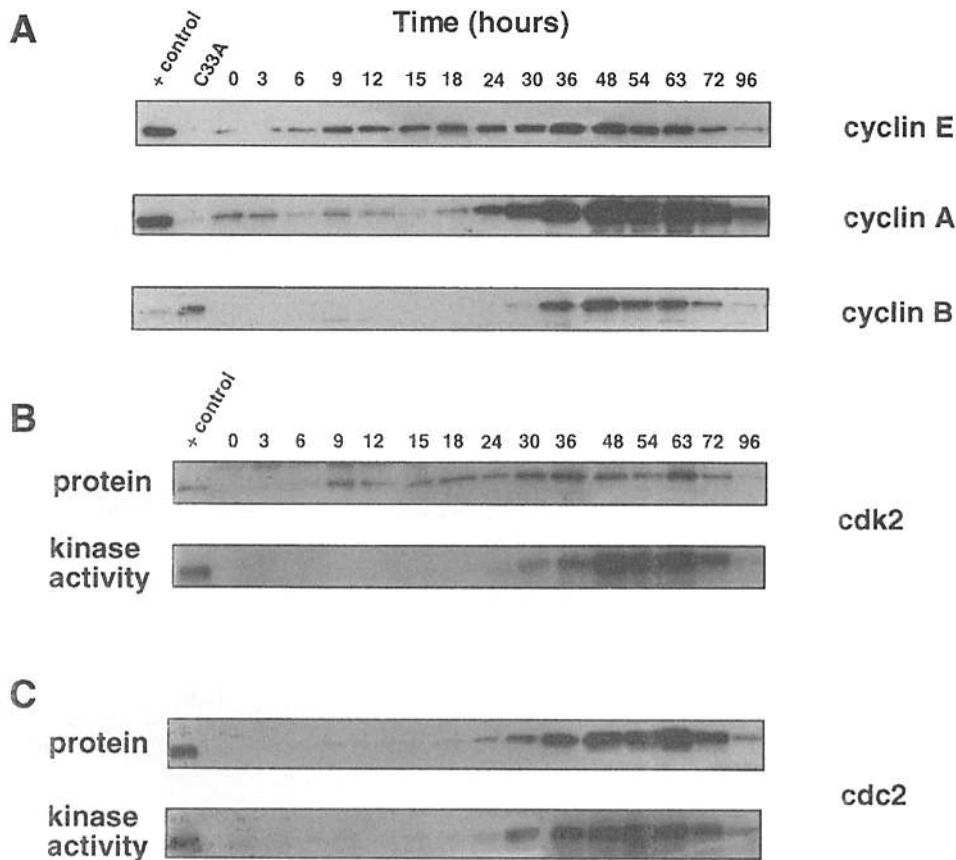


**Figure 2.** Induction of G1-associated cyclins, kinases, and Rb. Western blots were prepared from lysates of low density splenic B cells stimulated as described in Fig. 1. 125  $\mu$ g of whole cell lysates from each time point was loaded onto SDS-PAGE gels, along with lysates from P3X or NIH-3T3 murine (cyclin D1 only) or C33A human cells as positive controls. The gels were run for 1.5 h and transferred onto membranes for 24 h at 4°C. The blots were blocked, incubated in specific antibody preparations against cyclins D1, D2, D3 (A), cdk4, cdk6 (B), or Rb (C), followed by horseradish peroxidase-conjugated secondary antibodies. Enhanced chemiluminescence was performed and the blots were exposed to film for a 1-min initial exposure followed by a 15–30-min reexposure. The time in hours after stimulation is indicated. Similar results were obtained in a second independent experiment.

complex formation can be monitored via phosphorylation of the substrate histone H1 (49). The *in vitro* histone H1 kinase assay was performed on B cell lysates collected at the various time points. Specific cyclin/cdk complexes were immunoprecipitated using anti-cdk2 or anti-cdc2. Fig. 3 B demonstrates that, despite the presence of cdk2 early in the cell cycle, no kinase activity was detected until ~24 h after stimulation, i.e., immediately before the entry into S phase (see Fig. 1). In contrast, cdc2-associated kinase activity correlated with protein expression, with kinase activity being detectable just before S phase entry (Fig. 3 C). The peak of cdc2-associated kinase activity lagged behind the peak in cdk2-associated kinase activity by 6 h.

*Induction of Cell Cycle Inhibitors in B Cells Stimulated with Anti-Ig + IL-4.* The enzymatic activity of cyclin/cdk complexes can be modulated by a group of heterologous inhib-

itors (for a review see reference 46). Two inhibitors, p27 and p21, are broadly acting with reactivity toward a variety of cyclin/kinase complexes. As shown in Fig. 4 A, p27 is expressed at significant levels in unstimulated normal B cells. After stimulation of the B cells with anti-Ig + IL-4, the levels of p27 inhibitor decrease dramatically over the first 12 h, and reach their lowest level at 48 h (Fig. 4 A), a time point that correlates with the peak of [<sup>3</sup>H]thymidine incorporation (see Fig. 1). Expression of the p27 inhibitor is upregulated again after 48 h (Fig. 4 A), correlating with diminished DNA replication as well as an accumulation of cells arrested in G1 phase (see Fig. 1). Since p27 has been shown to mediate G1 arrest in overexpression systems (57), we suspected that the reexpression of p27 may contribute to the well documented G1 arrest that terminates B cell proliferation in response to anti-IgM stimulation (7, 8). To

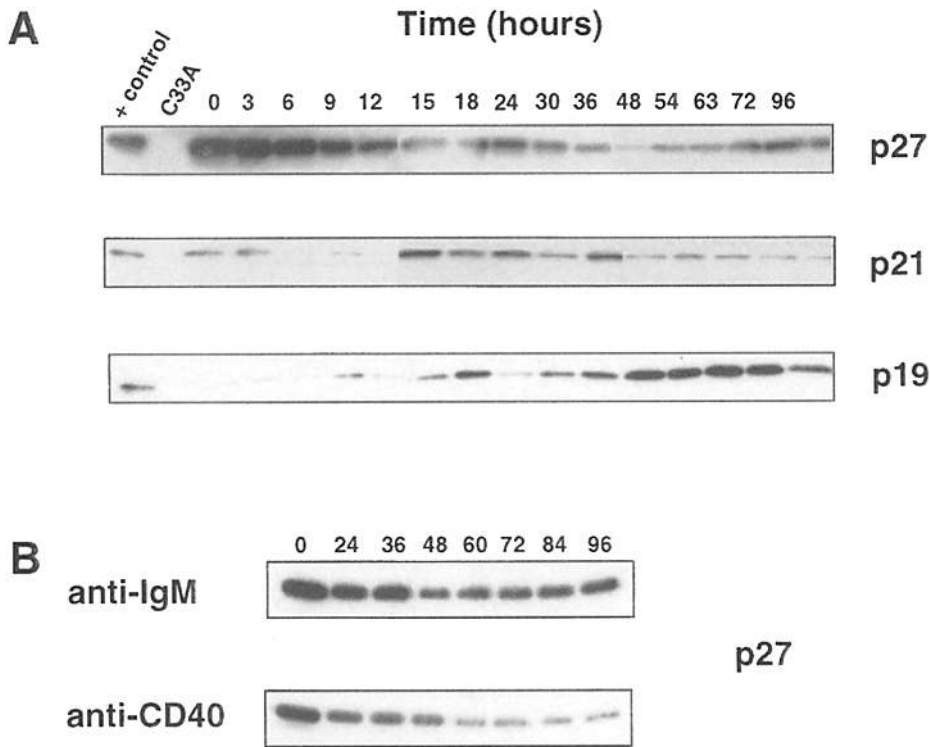


**Figure 3.** Induction of G1/S-, S-, and G2/M-associated cyclins, kinases, and kinase activities. Western blots of stimulated B cell lysates were incubated with specific antibodies against cyclins E, A, B (A), or cdc2 and cdk2 (B and C, *wp*), and processed as described. P3X or C33A cells were used as positive controls. The time in hours after stimulation with anti-Ig + IL-4 is indicated. 200  $\mu$ g of total cell lysates from B lymphocytes stimulated as described in Fig. 1 was used to perform in vitro kinase assays (B and C, *bottom*). cdk2- or cdc2-immunoprecipitated complexes were isolated, and kinase reactions were performed in 50 mM Hepes plus protease inhibitors, with the addition of 5  $\mu$ Ci  $\gamma$ -[ $^{32}$ P]ATP and 2.5  $\mu$ g histone per reaction as substrate. Reactions were performed at room temperature for 30 min, run on 12% gels, then dried and exposed to film for 3 h at room temperature. P3X cells were used as a positive control. The time in hours after stimulation is indicated.

evaluate this proposal, we compared the levels of p27 expression after antigen receptor cross-linking with the level of p27 expression in B cells stimulated with anti-CD40 antibodies, an activator capable of inducing long-term B cell proliferation (58). In these experiments, IL-4 was not used as a costimulant, since IL-4 delays the G1 arrest that follows anti-IgM stimulation. As seen in Fig. 4 B, p27 is only up-regulated after mitosis in B cells stimulated with anti-IgM and not in B cells stimulated with anti-CD40. B cells stimulated with the long-term stimulus anti-CD40 showed prolonged downregulation of p27 inhibitor (Fig. 4 B). These findings are consistent with the suggestion that p27 contributes to the G1 arrest that follows antigen receptor cross-linking.

Fig. 4 A also documents the expression of other cell cycle inhibitors in activated B cells. p21 is only weakly detectable at time zero, but is induced as cells enter cell cycle and found at highest levels corresponding to mid-G1 and G1/S interphase, i.e., from 15 to 36 h after stimulation. The INK4 family of inhibitors is specific for cdk4 and cdk6, thus affecting D-type complex activity (for a review see reference 46). Analysis of one such inhibitor, p19, showed that it is initially induced during late G1, with continued expression during S and G2/M phases, and can be detected at maximal levels by 48 h after stimulation (Fig. 4 A). The potential significance of these observations is discussed below.

*IL-4 Alone Causes Partial Induction of Cyclins D2 and D3 in Large B Cells.* As described above, IL-4 is a T cell-derived, viability-enhancing cytokine that potentiates anti-Ig-induced B cell proliferation (17), and truncates by  $\sim$ 12 h the G1 phase of cell cycle when IL-4-treated B cells are subsequently stimulated with anti-Ig (7, 28, 29). The above experiments characterize the cell cycle regulatory proteins induced in normal B cells stimulated with anti-IgM + IL-4. To determine the contribution that IL-4 may be making to these data, similar experiments were conducted comparing B cells stimulated with IL-4 alone, anti-Ig alone, or a combination of the two. The comparison was restricted to analysis of the G1-associated D cyclins, since the IL-4 stimulus does not drive B cells beyond the G1 phase of cell cycle. Low density B cells were stimulated with either 10  $\mu$ g/ml of anti-IgM or 100 U/ml of IL-4, and time points were collected as described above. The same cell preparation was also stimulated with anti-IgM + IL-4 for direct comparison. In this particular experiment, contrasting those above, trace amounts of cyclin D2 as well as the faster migrating species of D3 were detected in unstimulated cells (Fig. 5). Nevertheless, stimulation with either anti-Ig alone or anti-Ig + IL-4 produced a striking and rapid induction of cyclins D2 and D3 (Fig. 5, A and B), closely resembling the results shown previously in Fig. 2. In B cells cultured in IL-4 alone, cyclin D2 expression was induced with similar ki-



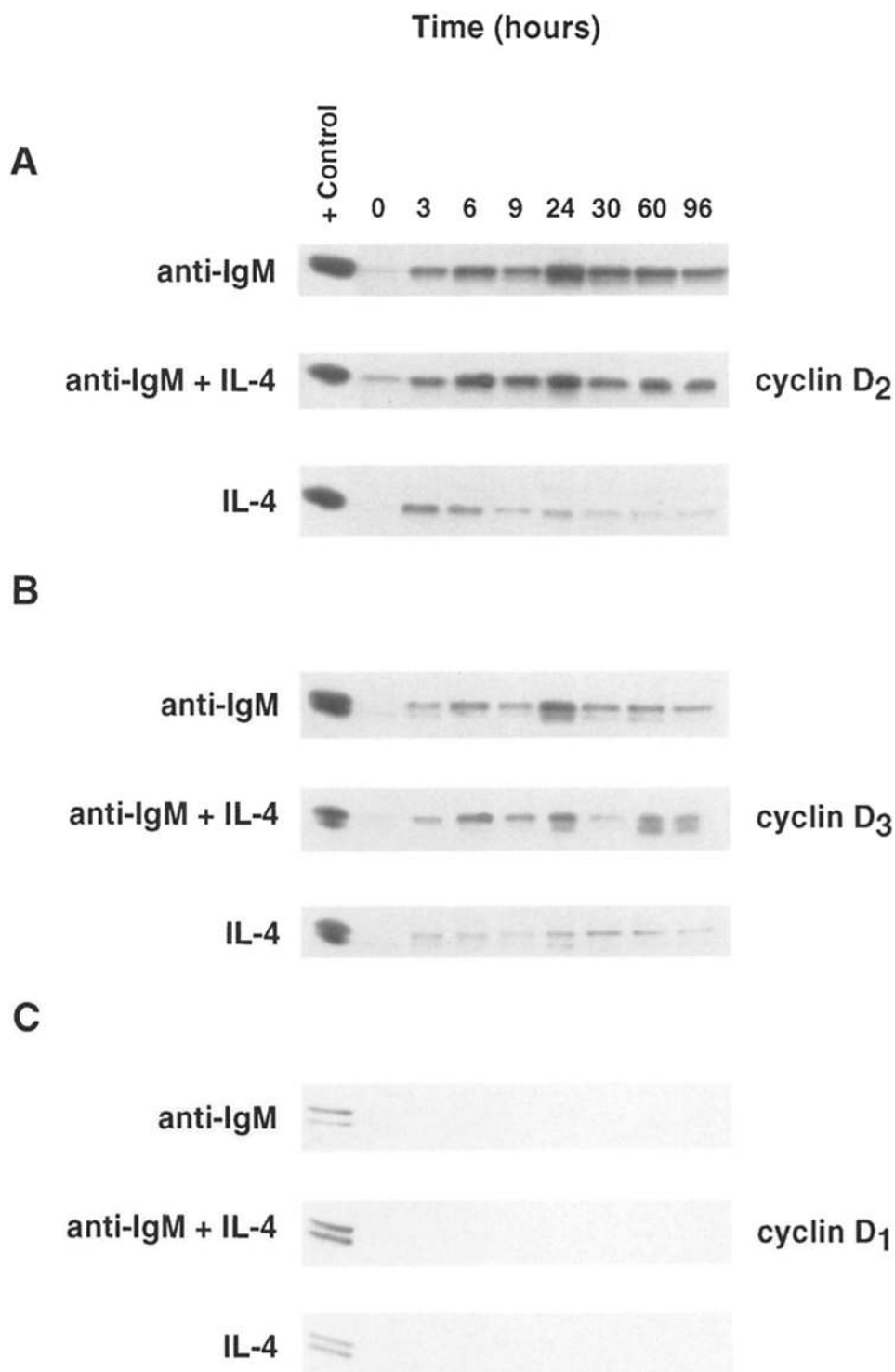
**Figure 4.** (A) Induction of cyclin-dependent inhibitors. Western blots were prepared as previously described, incubated with rabbit antisera against p27, p21, or p19 inhibitors, then processed as described. P3X was used as positive control. The time in hours after stimulation is indicated. Similar results were obtained in a second independent experiment. (B) Levels of p27 in anti-IgM- versus anti-CD40-stimulated B cells. B cells were stimulated for times indicated with anti-IgM (25  $\mu$ g/ml) or anti-CD40 (25  $\mu$ g/ml) in the absence of IL-4. Western blots were prepared as described in Fig. 2. Blots were incubated with rabbit anti-p27 antibody and processed as described. Similar results were obtained in a second experiment.

netics, i.e., by 3 h; however, the induction was notably transient in nature with levels reduced by 6 h (Fig. 5 A). Prolonged but weak cyclin D3 expression was observed (Fig. 5 B). Cyclin D1 was not detected under any stimulation conditions (Fig. 5 C). These data indicate that IL-4 causes suboptimal induction of cyclins D2 and D3, but does not achieve the sustained response obtained after anti-Ig stimulation.

### Discussion

Antigen-specific clonal expansion of B lymphocytes is a critical component of antibody-mediated humoral immunity. The *in vivo* expansion of antigen-specific B cells converts an initial B cell precursor frequency of  $\sim 1$  in  $10^5$  (1) to a greatly expanded pool of cells in which individual precursors have divided to as many as  $10^4$  daughter cells (for a review see reference 59). To extend our understanding of how this extensive clonal expansion is regulated, the current study explores the induction of cell cycle regulatory proteins, including cyclins, cdk, and cdk inhibitors, in B cells stimulated *in vitro* specifically at their antigen receptor using anti-Ig antibodies. This stimulus drives all B cells, irrespective of their antigen specificity, to enter the G1 phase of cell cycle (5, 6), with  $\sim 50$ – $60\%$  continuing on to complete the cell cycle and divide (5, 6), and then arrest in the G1 phase of the subsequent cycle (7, 8). To minimize the effects of dying cells throughout the extended 96-h culture period, anti-Ig-stimulated B cells were cocultured with IL-4, a viability-enhancing cytokine (19–22) that additionally

truncates the G1 phase of cell cycle of anti-Ig-stimulated B cells (7, 28, 29). Our data indicate that the type and sequence of cell cycle regulatory proteins expressed in B lymphocytes after stimulation with anti-Ig + IL-4 was similar to those characterized in other cell systems, e.g., fibroblasts or macrophages (for reviews see references 37, 38). B cells activated in this manner show a striking and synchronous induction of cyclins D2 and D3, but not D1, occurring as rapidly as 3 h after stimulation, above the low or undetectable amounts expressed in unstimulated large B cells. An indirect indication of D-associated kinase activity can be obtained by examining the extent of phosphorylation of endogenous Rb, the presumed target of cyclin D-associated kinase activity *in vivo* (51–54). Surprisingly, the phosphorylation of Rb, initially detected in our experiments at 18 h, lagged behind the induction of cyclins D2 and D3 and their associated kinases by at least  $\sim 9$  h (Fig. 2). Thus, despite the expression of all components required for effective cyclin/kinase complex formation at 9 h after stimulation, no kinase activity detectable by endogenous Rb phosphorylation was seen until late G1 phase (i.e., 18 h after stimulation). This apparent lag in kinase activation may reflect a delay in the activation of the complex either by incomplete assembly or lack of the cdk-activating kinase, CAK (60). Alternatively, the complexes may be blocked by inhibitors, a proposal that is consistent with the appearance of p19 and p21 inhibitors  $\sim 12$  h after stimulation with anti Ig + IL-4 (Fig. 4). As a final consideration, it is possible that Rb phosphorylation does not accurately reflect D kinase activity in B lymphocytes.



**Figure 5.** Induction of G1-associated cyclins by anti-IgM alone or IL-4 alone.  $10^6$  low density B cells/ml were stimulated with 10  $\mu$ g/ml anti-IgM plus 100 U/ml IL-4, 10  $\mu$ g/ml anti-IgM alone, or 100 U/ml IL-4 alone. An abbreviated time course was performed with lysates prepared at 0, 3, 6, 10, 24, 30, 60, and 96 h after stimulations. 125  $\mu$ g of total cell lysate from each time point was run on SDS-PAGE, and Western blots were prepared as described in Fig. 2. Blots were incubated with specific antibodies against cyclins D2 (A), D3 (B), and D1 (C), and processed as described above. P3X cells were used as a positive control for cyclins D2 and D3. NIH-3T3 cells were used as a positive control for cyclin D1. The time in hours after stimulation is indicated.

In addition to these detailed analyses of G1 phase events in anti-Ig-stimulated B cells, our experiments also evaluate the appearance of cell cycle regulatory proteins controlling the later phases of the anti-Ig-induced B cell cycle. The expression of cyclin E and its kinase partner cdk2 was readily evident by mid G1 phase of the anti-Ig-induced B cell cycle (Fig. 3), consistent with other reports (39). How-

ever, we could not detect cdk2-associated kinase activity using the histone substrate assay until cells had entered the S phase of cell cycle, consistent with reports from Tanguay and Chiles (61). This delay in kinase activation beyond the expression of both cyclin and kinase protein was particularly surprising, since cyclin E/cdk2-associated kinase activity has been shown in other systems to act earlier in the



cell cycle, controlling transition from G1/S (39, 40). One possible explanation for the delayed appearance of cdk2 activity is the observed induction of the inhibitor p21 at 15 h after stimulation (Fig. 4), since it has been shown in other cell systems that p21 inhibitor can be precipitated in complexes of cyclin E/cdk2, and, under appropriate conditions, leads to inhibition of cdk2 kinase activity (62, 63). In B cells stimulated with anti-Ig + IL-4, cyclins A and B, and their preferred kinase partner cdc2, were expressed at either the G1/S interphase (cyclin A and cdc2), or during S phase (cyclin B) (Fig. 3). In contrast to the delayed activation of cdk2, activation of cdc2 kinase activity correlated with protein expression (Fig. 3), in agreement with earlier reports on mitogen-stimulated human B cells (64).

The data described in this report represent one of the few analyses to date describing cell cycle regulatory proteins in activated nontransformed lymphocytes. It provides a comprehensive evaluation of the full complement of cyclins and cdk, as well as several of the cdk inhibitors, in activated primary B lymphocytes. Our work compliments that of Palermo et al. (65), who previously investigated a panel of human B lymphomas and found that they expressed cyclins D2 and/or D3, however, most lacked cyclin D1 expression. The few that did express cyclin D1, in fact, exhibited the t(11;14), or bcl-1 translocation (66, 67), a chromosomal abnormality involving translocation of cyclin D1 on chromosome 11 to the Ig heavy chain locus on chromosome 14. The pattern of D cyclin regulatory proteins observed in anti-Ig-stimulated nontransformed B cells (e.g., Figs. 2 and 5) is similar to that expressed by nontransformed T lymphocytes after stimulation with mitogens (68, 69). However, this lymphoid pattern of D cyclin expression is not typical of all hemopoietic cells. Considerable work has focused on the expression of cell cycle regulatory proteins in activated macrophages, and in this lineage, the cells express cyclins D1 and D2, but not cyclin D3 (70). Since little is known regarding the functional differences

between the D-type cyclins, the significance of their lineage-restricted combinatorial expression is not understood.

The current study provides further insight into the mechanism by which IL-4 augments anti-Ig-induced B cell proliferation. This phenomenon has indeed represented somewhat of a conundrum to date, since IL-4 is neither a growth factor per se, nor an obligatory competence factor. In dissecting the effect of this cytokine on the anti-Ig-induced B cell proliferative response, Rabin et al. (7, 28) have elegantly shown that IL-4, while incapable of driving B lymphocytes into S phase, dramatically truncates the G1 phase of cell cycle. This property of IL-4 may well be explained by our data showing that IL-4 induces transient and weak expression of cyclins D2 and D3, respectively, in primary B lymphocytes receiving no other exogenous stimulus (Fig. 5), since previous studies in other cell types have clearly demonstrated that D cyclin induction is rate limiting in the progression to S phase (40, 41).

Our data also provide a plausible molecular explanation for the previously documented G1 arrest that follows the initial proliferation of primary B cells in response to anti-Ig stimulation (8). In other cell systems, G1 arrest is thought to be mediated at least in part by certain inhibitory molecules such as p27 (for a review see reference 46) with overexpression of p27 effectively causing G1 arrest in a variety of cell types (57, 71). As shown in Fig. 4, the reexpression of p27 at later time points with the exit of activated B cells from cell cycle and correlates with an accumulation of cells in G1 phase (Fig. 1). In contrast, p27 was destroyed without subsequent reexpression after stimulation of B lymphocytes with anti-CD40, a stimulant capable of inducing long-term B cell proliferation (Fig. 4 B). These data are consistent with the notion that G1 arrest of anti-Ig-stimulated B cells after the initial round of replication may be mediated by p27 and, conversely, that the ability of anti-CD40 to mediate long-term proliferation may be related to its ability to induce permanent downregulation of p27.

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We thank Dr. Charles Sherr for his generosity in sharing detailed procedures and antibodies against p19 and cdk4; Drs. Hideo Toyoshima and Tony Hunter for supplying antibodies against p27, and Dr. Martin McMahon (DNAX) for valuable discussions.

DNAX Research Institute is fully funded by Schering Plough.

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Received for publication 27 November 1995 and in revised form 10 May 1996.

Note added in proof: Since the submission of this manuscript, Tanguay and Chiles have reported similar findings using small dense B cells (Tanguay, D.A., and T.C. Chiles. *J. Immunol.* 156:539-548).

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