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Role of Cyclin A and p27 in Anti-IgM–induced G_1 Growth Arrest of Murine B-Cell Lymphomas

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Cross-linking surface immunoglobulin (Ig)M on the WEHI-231 B-cell lymphoma results in decreased cell size, G_1/S growth arrest, and finally DNA cleavage into oligonucleosomal fragments that are the classical features of apoptotic cells. Treatment of WEHI-231 cells with anti-IgM in early G_1 phase prevents phosphorylation of the retinoblastoma gene product (pRb) and inhibits entry into S phase. Using unsynchronized cells, we previously demonstrated that cyclin A–associated and Cdk2-dependent GST-pRb kinase activity were inhibited in WEHI-231 cells treated with anti-IgM. We now show that progression of elutriated early G_1 phase WEHI-231 cells from early into late G_1 phase is accompanied by an increase in the abundance of cyclin A protein and cyclin A–associated kinase activity. Treatment of early G_1 cells with anti-IgM prevented this increase in cyclin A–associated kinase activity at late G_1, despite minimal changes in the overall level of cyclin A and Cdk2 proteins. Late G_1 cells, which already possess high cyclin A–associated kinase activity, were insensitive to anti-IgM treatment and were able to complete the cell cycle. We also found that anti-IgM–treated cells contained increased amounts of the Cdk inhibitor protein $p27^{Kip1}$. Essentially all of the cyclin A in treated cells was associated with p27, a result which we propose explains the lack of cyclin A/Cdk2 kinase activity. Accumulation of p27 in cyclin A kinase complexes, however, did not decrease the amount of Cdk2 bound to cyclin A. Thus, cross-linking IgM on growth-inhibitable B-cell lymphomas affects cyclin A kinase activity by increasing the levels of p27 in this complex, thus preventing productive pRb phosphorylation and leading to cell cycle arrest and subsequent apoptosis. These results are discussed in terms of the cell cycle restriction points that regulate lymphocyte function, as well as the lineage-specific differences in cell cycle control.

INTRODUCTION

The WEHI-231 lymphoma has been used as a model for immature B-cell tolerance based on its phenotype ($sIgM^{high}$, $sIgD^{low}$) and exquisite sensitivity to anti-Ig treatment (Scott, 1993). That is, cross-linking of surface IgM receptors on WEHI-231 cells induces cell cycle blockade, followed by programmed cell death (Benhamou *et al.*, 1990; Hasbold and Klaus, 1990). Despite the rapidly accumulating data on signal transduction through the immunoglobulin (Ig) receptor complex, the growth inhibitory intracellular signaling pathways still need to be elucidated. We know that treatment of WEHI-231 cells with anti-IgM leads to an accumulation of viable cells in late G_1 (Scott *et al.*, 1986). Once cells become arrested, they appear to be committed to apoptosis: cell viability decreases gradually during the next 24–48 h (Fischer *et al.*, 1994). We have previously shown that delivery of a negative signal must occur in early G_1 because delayed addition of anti-IgM (to late G_1 cells) does not block cell cycle progression and exposure to anti-IgM during S phase fails to inhibit

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transition through DNA synthesis and mitosis (Scott *et al.*, 1986). This change in the sensitivity of cells to the growth-suppressing effects of anti-IgM does not depend on early membrane events because the level of expression of sIgM and calcium mobilization upon IgM cross-linking remain the same in different phases of the cell cycle (Scott *et al.*, 1987). Hence, the availability or responsiveness of intracellular target(s), lying downstream of membrane receptor cross-linking, might restrict sensitivity to early G_1 signaling.

We and others subsequently demonstrated that anti-IgM treatment leads to the accumulation of the hypophosphorylated form of the retinoblastoma gene product pRb (Maheswaran *et al.*, 1991; Warner *et al.*, 1992), a process which only occurs when anti-IgM is added to cells in early G_1 (Joseph *et al.*, 1995). The product of the retinoblastoma gene is a nuclear phosphoprotein, which has been shown to undergo cell cycle–dependent phosphorylation and dephosphorylation (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989; Mihara *et al.*, 1989; Ludlow *et al.*, 1990, 1993; Durfee *et al.*, 1993). Hypophosphorylated pRb is growth repressive and is tightly associated with the nucleus (Mittnacht and Weinberg, 1991; Templeton, 1992). The hypophosphorylated form of pRb regulates cell cycle progression by binding a number of proteins, including E2F. Upon phosphorylation, E2F is released and the cells progress into S phase (Ewen, 1994; La Thangue, 1994).

Phosphorylation of pRb on serine and threonine residues in G_1 is presumably dependent on the formation of an active cyclin-Cdk complex. At least three kinase complexes, including cyclins A-, E-, and D-type, can phosphorylate pRb in vitro (Lees *et al.*, 1991; Akiyama *et al.*, 1992; Hu *et al.*, 1992; Matsushime *et al.*, 1992; Meyerson and Harlow, 1994), but the question of which cyclin-Cdk complex phosphorylates pRb in vivo is unanswered. The three D-type cyclins have been implicated in the regulation of G1 progression (Hunter and Pines, 1994; Sherr, 1994), whereas cyclins A and E have been shown to participate in the G_1 to S transition (Girard *et al.*, 1991; Koff *et al.*, 1992; Lees *et al.*, 1992; Pagano *et al.*, 1992; Rosenblatt *et al.*, 1992; Zindy *et al.*, 1992; Tsai *et al.*, 1993). The fact that cyclin D is not essential in cells lacking pRb suggests that the cyclin D-Cdk4/6 complex is important in pRb phosphorylation in vivo. Earlier reports that cyclin A protein accumulates only from S phase onward (Pines and Hunter, 1992; Zindy, *et al.*, 1992) is not universally true because in certain other cell models, cyclin A message and proteins are expressed in G_1 before the onset of DNA replication (Bybee and Thomas, 1992; Bui *et al.*, 1993; Carbonaro-Hall *et al.*, 1993). Importantly, induction of cyclin A expression in G_1 has been noted in normal human T cells upon the stimulation of the T-cell receptor alone, a treatment that is not sufficient to promote S-phase entry (Firpo *et al.*, 1994).

Among the Cdks, direct evidence has been obtained for the participation of Cdk2 in G_1/S transition (Tsai *et al.*, 1993; van den Heuvel and Harlow, 1993). On the other hand, the ectopic expression of cyclin A and E, the activating partners of Cdk2, can overcome pRb-mediated growth arrest in G_1 by inducing pRb hyperphosphorylation (Hinds *et al.*, 1992). Because anti-IgM prevents phosphorylation of pRb in WEHI-231 cells only when added in early G_1 (Joseph *et al.*, 1995), and leads to growth arrest near the G_1/S border without affecting the duration of G_1 (Page and DeFranco, 1990), we set out to determine whether negative signaling affects the accumulation and activity of cyclin A or cyclin E–associated kinase complexes. Although cyclin A proteins were easily detected in lysates of WEHI-231 cells by immunoblotting and immunoprecipitation, the level of cyclin E expression was very low even in exponentially growing cells, as reported recently for murine splenic B-cells (Tanguay and Chiles, 1994). This probably reflects a lineage-specific dependence for cyclin E expression. Nonetheless, as described herein, anti-IgM treatment modulated cyclin A-Cdk2 (and cyclin E-dependent kinase) complexes, but only if added before a critical point in early/mid G_1, to cause growth arrest in late G_1 leading to apoptosis. Inhibition of cyclin A kinase activity is due to an increase in the expression and association of the Cdk inhibitor $p27^{Kip1}$, and results in decreased phosphorylation of pRb.

MATERIALS AND METHODS

Cell Lines and Culture

The WEHI-231 murine B-cell lymphoma was maintained in complete media (RPMI-1640 with 5% fetal calf serum [FCS]), as described earlier (Scott *et al.*, 1986, 1987; Fischer *et al.*, 1994). The murine 3T6 fibroblast cell line was maintained in DMEM (Bio-Whittaker, Walkersville, MD) supplemented with 5% FCS (Sigma, St. Louis, MO), 2 mM [l]-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

For growth inhibition assays, WEHI-231 cells were incubated for 24 h at 5×10^5 cells/ml with 1.0 μg/ml goat anti-mouse polyclonal anti-IgM (μ chain-specific, Jackson ImmunoResearch Laboratories, West Grove, PA).

Centrifugal Elutriation

WEHI-231 cells were elutriated to obtain early and late G_1 cells essentially the same as described elsewhere (Joseph *et al.*, 1995). Cell size distribution was measured with a Coulter Channelyzer system (Coulter Electronics, Hialeah, FL) and the median cell size of a given cell fraction, together with analysis of DNA content, was used to verify the position of cells in the cell cycle.

Propidium Iodide Staining and Cell Cycle Analysis

One million cells from elutriated fractions or unsynchronized cell culture were washed three times with phosphate-buffered saline (PBS) and fixed in 70% ice-cold ethanol. After 24 h, cells were washed again with PBS and incubated with RNase at a final concentration of 1 μg/ml at 37°C for 30 min; propidium iodide was then added at a final concentration of 10 μg/ml. Stained cells were

analyzed on a FACScan flow cytometer. A cell cycle analysis algorithm was used to calculate the relative amount of cells in G_1, S, and G_2/M phases.

Antibodies, Immunoprecipitation, and Kinase Assay

Rabbit anti-human cyclin A antiserum was the generous gift of Dr. Konstantin Galaktionov (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Rabbit anti-human Cdk2 antiserum was purchased from Upstate Biotechnology (Lake Placid, NY), and purified anti-Cdk4 and cyclin D2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-human retinoblastoma protein (Mh-rb-02) was obtained from PharMingen (La Jolla, CA). Rabbit anti-cyclin D1 antiserum was kindly provided by Dr. Charles Sherr (St. Judes Research Center, Memphis, TN). Anti-$p27^{Kip1}$ was prepared in rabbits against full length murine p27 purified after cleavage from bacterially expressed GST-p27 (Toyoshima and Hunter, 1994).

WEHI-231 cells were labeled with 200 μCi/ml of [^{35}S]methionine, cysteine (EXPRE^{35}S^{35}S, Protein Labeling Mix, DuPont, Boston, MA) in methionine- and cysteine-free medium supplemented with dialyzed FCS for 4 h. Cells were then washed with ice-cold PBS and lysed in NP-40 buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 50 mM NaF, 0.5 mM sodium orthovanadate, 20 mM sodium pyrophosphate, 10 mg/ml aprotinin and leupeptin, and 1 mM PMSF). Lysates were clarified by centrifugation at 10,000 × *g* for 20 min. Supernatants (about 1 mg of total cellular proteins) were precleared for 2 h at 4°C with Sepharose beads and protein A-Sepharose beads. This was followed by overnight immunoprecipitation at 4°C with protein A-Sepharose beads (10 μl) precoated with anti-cyclin A or anti-Cdk2 antibodies (rabbit antiserum 1 μl/10 μl beads). Immunocomplexes collected on protein A-Sepharose were washed four times with lysis buffer and boiled for 10 min in 50 μl of Laemmli sample buffer. Samples were electrophoresed through a 10% SDS-polyacrylamide gel. After fixation, gels were treated with autoradiography enhancer (EN^{3}HANCE™, DuPont), dried, and exposed to X-ray film (BioMax MR, Kodak, Rochester, NY). For re-immunoprecipitation, cyclin A and Cdk2 immunoprecipitates were boiled in sample buffer, cooled on ice, diluted with lysis buffer up to 0.1% of SDS, and incubated with protein A-Sepharose to eliminate anti-Cdk2 and anti-cyclin A antibodies. Re-immunoprecipitation was then performed using anti-cyclin A antibodies as described.

For kinase reactions, immunoprecipitates were washed four times with lysis buffer and once with kinase buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.0, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 2 μM unlabeled ATP). The beads were suspended in 20 μl kinase buffer containing 2 μg of soluble glutathione *S*-transferase-pRb fusion protein (residues 379–928; the construct for which was the kind gift of Dr. John Ludlow, University of Rochester, NY), and 20 μCi of [γ-^{32}P]ATP (NEN; 3000 Ci/mmol) and incubated at room temperature for 30 min. The reaction was stopped by addition of 20 μl 2× SDS-sample buffer. After boiling for 10 min, samples were electrophoresed in polyacrylamide gels and the phosphorylated pRb was revealed by autoradiography.

Immunoblotting

Cells were prepared and lysed as described previously (Joseph *et al.*, 1995). Briefly, 3 × 10^6 cells were lysed in SDS stop buffer, immediately boiled, and electrophoresed in a 10% gel at constant current. Proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA). The membranes were blocked with 2% bovine serum albumin in Tris-buffered saline with 0.2% Tween 20 for 2 h at room temperature and incubated with primary antibodies at 1:400 for anti-human pRb (Mh-rb-02; PharMingen) or 1:1000 (anti-cyclin A and anti-Cdk2 rabbit antiserum) for 1–2 h at room temperature. Primary antibodies are followed by appropriate secondary antibodies coupled to alkaline phosphatase (Fisher Scientific, Pittsburgh, PA). Nitroblue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) were used for visualization of the bands.

RESULTS

Kinetics of Growth Arrest and pRB Phosphorylation

We have shown previously that cells in the early G_1 phase of the cell cycle (G_{1A}) are sensitive to growth inhibitory signals generated by anti-IgM treatment, whereas cells from other phases (G_{1B}, S, and G_2/M) are not. The latter populations complete the cell cycle in the presence of anti-IgM at the same rate as control cells until they reach early G_1 in the next cycle. Thus, the duration of treatment needed to observe growth arrest should reflect the length of time required for cells to progress from early G_1 to the restriction point in late G_1. To confirm this, we cultured exponentially growing WEHI-231 cells in the presence of anti-IgM (1 μg/ml) and analyzed them by flow cytometry at different time points to determine the number of cells in G_1 and S. We also used immunoblotting followed by densitometric analysis to estimate the ratio between hypo- and hyperphosphorylated forms of pRB and considered bands migrating faster than the major slow band as hypo-pRb (Figure 1). During the first 10–12 h of treatment, the rate of accumulation of cells in G_1 was very low, but increased during the next 12 h, in concert with the appearance of the hypophosphorylated form of pRB. By 24 h of treatment with anti-IgM, about 70% of pRb protein exists in the hypophosphorylated form, although a slowly migrating (hyperphosphorylated) form still can be detected. This pattern of pRb phosphorylation is specific for G_1-arrested cells, and becomes apparent in purified early G_1 cells treated with anti-IgM in 4–7 h (Joseph *et al.*, 1995; see below).

Because multiple G_1 cyclins are involved in the functional inactivation of pRb by hyperphosphorylation (Hatakeyama *et al.*, 1994), the appearance of the partially phosphorylated form of pRb may result from inactivation of some of the cyclins and/or Cdks, presumably those responsible for the G_1 to S transition. Direct evidence has been obtained only for the participation of Cdk2 in $G_1 \rightarrow$ S transition (Tsai *et al.*, 1993; van den Heuvel and Harlow, 1993). Thus, it is interesting that the ectopic expression of Cdk2 partners in the kinase reaction, cyclins A and E, can overcome pRb-mediated growth arrest in G_1 by inducing pRb hyperphosphorylation (Hinds *et al.*, 1992). This suggests that complexes containing Cdk2 and cyclin A (or cyclin E) may function in pRb phosphorylation. This was investigated next.

Anti-IgM Treatment of Early G_1 WEHI-231 Cells Results in Inhibition of Cyclin A–associated Kinase Activity

As we have shown previously (Joseph *et al.*, 1995), cyclin A–associated and Cdk2-dependent GST-pRb ki-

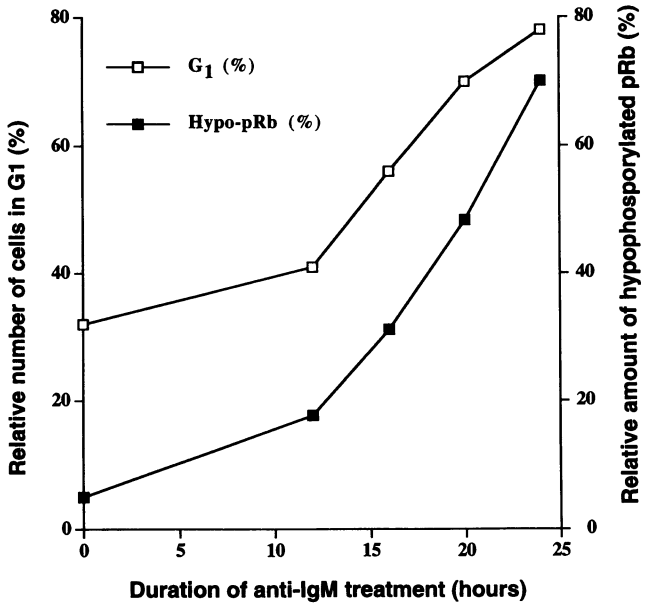

Figure 1. Kinetics of growth arrest by anti-IgM and status of pRb phosphorylation in the WEHI-231 B-cell lymphoma. Cells were cultured in the presence or absence of anti-IgM and the state of pRb phosphorylation in each sample was determined by immunoblotting of whole cell lysates probed with anti-human pRb monoclonal antibodies, followed by densitometry. Hypo-pRb refers to the bands that migrated faster than the major, slow pRb band. The percentage of cells in G_1 was calculated using flow cytometry of propidium iodide–labeled cells. The appearance of hypophosphorylated, growth suppressive form of pRb correlates with cellular accumulation in G_1.

nase activity is diminished in anti-IgM–treated WEHI-231 cells, a result that may explain the prevention of pRb phosphorylation and cell cycle arrest. If our model is correct, then anti-IgM treatment of early G_1, but not late G_1, cells should result in inhibition of cyclin A activity. To determine whether the cyclin A-Cdk2 complex is a target for anti-IgM per se, we separated WEHI-231 cells into early and late G_1 fractions and cultured them for up to 7 h in the absence or presence of anti-IgM. Figure 2 shows that cyclin A–associated GST-pRb kinase activity was low in early G_1 cells, but was high in late G_1 fractions. When anti-IgM was added at time zero to early G_1 cells, cyclin A–associated GST-pRb kinase activity was clearly inhibited at 7 h, but addition of anti-IgM to late G_1 cells or to early G_1 cells after a 4-h delay (Ezhevsky and Scott, unpublished results) did not reduce the level of cyclin A–associated kinase activity 7 h later. Thus, anti-IgM treatment prevents the activation of a cyclin A/Cdk2 complex that occurs well before the G_1/S boundary, but does not change the activity of this complex once it has formed. This observation readily explains the sensitivity of early G_1 WEHI-231 cells to arrest by anti-IgM treatment.

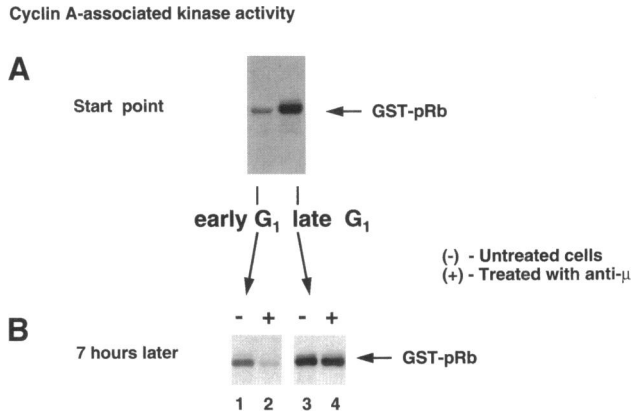

Figure 2. Anti-IgM treatment results in inhibition of cyclin A–associated GST-pRb kinase activity in early G_1 cells, but does not affect late G_1 WEHI-231 lymphoma cells. (A) Cyclin A–associated kinase activity in elutriated early and late G_1 cells. (B) Effect of anti-IgM treatment on cyclin A–associated GST-pRb kinase activity in early and late G1 cells. Elutriated early and late G_1 cells were incubated with (+) or without (−) anti-IgM for 7 h and the in vitro kinase assays were performed on cyclin A immunoprecipitates, using GST-pRb as a substrate.

Effect of Anti-IgM on Cyclin A and Cdk2 Protein Expression in G_1

To determine whether the level of expression of cyclin A/Cdk2 proteins is affected by anti-IgM, we performed immunoblots of whole cell lysates of elutriated WEHI-231 cells before and after treatment with anti-IgM. As shown in Figure 3 (left panel), the abundance of cyclin A protein was very low in elutriated early G_1 cells, as expected. By late G_1, the level of cyclin A increased (Figure 3, center panel) and an additional, slow migrating form of cyclin A appeared. This form could be recognized by rabbit anti-cyclin A antibodies in lysates of selected murine, but not in human cells (Ezhevsky and Scott, unpublished results). As we have shown before, the appearance of the slow migrating form of cyclin A correlates with the high level of cyclin A–associated kinase activity in WEHI-231 cells (Joseph *et al.*, 1995), but its identity is unknown.

When anti-IgM was added to early G_1 cells at time zero, the level of expression of the major cyclin A form did not change, but the appearance of the slow migrating form of cyclin A was blocked concomitant with the lack of kinase activity (see above). In agreement with our previous data (Joseph *et al.*, 1995), phosphorylation of pRb is also inhibited in these synchronized lymphoma cells. Late G_1 cells, which already express both forms of cyclin A, partially phosphorylated pRb, and high kinase activity, were insensitive to the anti-IgM treatment and modification of cyclin A (Figure 3, center panels). As expected, when late G_1 cells were treated for an additional 14 h, they were able to complete the cell cycle and became

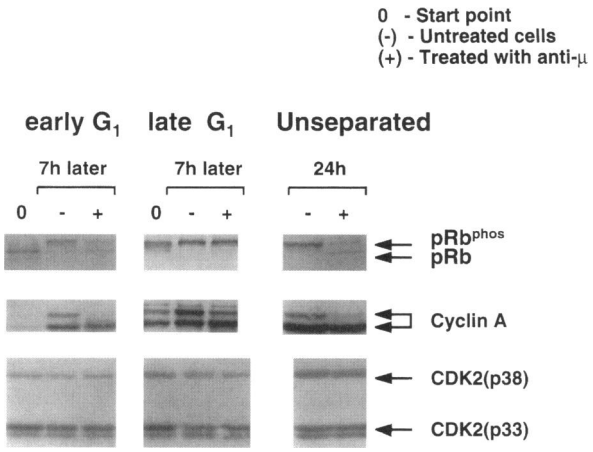

Figure 3. Pattern of cyclin A, Cdk2 protein expression and state of pRb phosphorylation in early and late G_1 WEHI-231 cells before and after anti-IgM treatment. Counterflow centrifugal elutriation was used for the separation of early (left panels) and late (center panels) G_1 WEHI-231 cells. Cells were then re-cultured for 7 h in complete media with (+) or without (−) anti-IgM, lysed in SDS-sample buffer, and total cell lysates were probed with anti-pRb (upper panels), anti-cyclin A (middle panels), or anti-Cdk2 (lower panels) for immunoblotting. Note that the lower band of the 33-kDa doublet was not detected in anti-cyclin A immunoprecipitates and its identity is unknown (see Figure 10). Unseparated WEHI-231 cells, treated with anti-IgM for 24 h, were used as a control (right panels).

arrested after they re-entered the G_1 phase. At that time, the pattern of cyclin A expression and pRb phosphorylation were exactly the same as in the case of early G_1 cells after 7 h of treatment (our unpublished results).

The level of Cdk2 expression did not change under any of the above conditions (Figure 3, lower panels). On immunoblots of whole cell proteins, the anti-Cdk2 antibodies detected a doublet at ~33 kDa (the lower band is not detected in anti-cyclin A immunoprecipitates–see Figure 10–and its identity is unknown), as well as an additional band of ~38 kDa, which is the murine-specific form of Cdk2 originating from the alternative splicing of the same gene (Yasuda *et al.*, 1993). Based on these observations, we conclude that the negative effects of sIgM cross-linking on early G_1 cells are due in part to modification of the cyclin A-Cdk2 complex.

Because the pattern of appearance of these proteins, their associated kinase activities, and the status of pRb phosphorylation were the same as in unseparated WEHI-231 cells treated with anti-IgM for 24 h (compare left and right panels in Figure 3), in the following experiments, we utilized unseparated WEHI-231 lymphoma cells, treated for 24 h with anti-IgM, as representative of the arrest seen in early G_1 cells. We first examined cyclin E–associated kinase activity because this cyclin is a known partner of Cdk2 (Koff *et al.*, 1993; Pagano *et al.*, 1993). These experiments demonstrated that cyclin E–associated kinase activity was also reduced by a 24-h treatment of WEHI-231 cells with anti-IgM (Figure 4). The abundance of cyclin E in our murine B-lymphoma cells is very low; therefore, to detect cyclin E–associated kinase activity, an exposure time of 8 h was needed, compared with 5 min used in the cyclin A kinase reaction.

Anti-IgM Increases the Amount of p27 Associated with the Cyclin A Complex

One explanation for the lack of cyclin A/E-Cdk2 kinase activity is that a Cdk inhibitor was present in anti-IgM–arrested cells. However, when lysates from anti-IgM–treated and untreated WEHI-231 cells were mixed, cyclin A– and cyclin E–associated kinase activity was not decreased (Figure 4, right lanes). This suggests that Cdk inhibitors, if present, were not in excess and existed in a tight complex with cyclin A/E or Cdk2. We predicted, however, that the amount of inhibitor associated with the cyclin complexes should increase in proportion to the total amount of inhibitor present during growth inhibition. Our preliminary experiments showed that cyclin A co-precipitated with an ~27-kDa protein, and the amount of this protein increased in cyclin A precipitated in anti-IgM–arrested cells (Scott *et al.*, 1995). Surmising that this protein might be the $p27^{Kip1}$ Cdk inhibitor, which is expressed at high levels in growth-arrested T cells (Nourse *et al.*, 1994) and appears as a complex with cyclins A/E-Cdk2 or cyclin D-Cdk4 kinases (Kato *et*

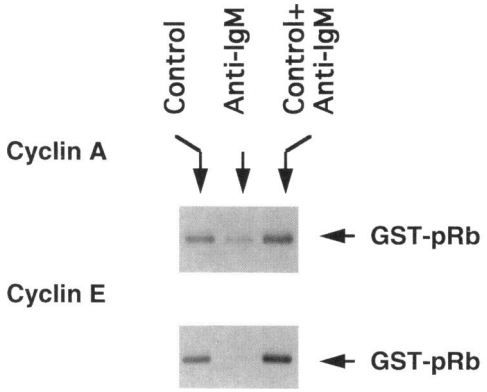

Figure 4. Effect of anti-IgM on cyclin A– (top panel) and cyclin E–associated kinase activity (bottom panel). Extracts of control WEHI-231 cells and those treated with anti-IgM for 24 h were immunoprecipitated with rabbit antiserum to human cyclin A or anti-mouse cyclin E. Immune complexes were collected on protein A-agarose beads and assayed for GST-pRb kinase activity (as in Figure 2). In the right lane of each set, mixtures of treated and untreated WEHI-231 lysates were immunoprecipitated and assayed for excess Cdk inhibitor activity. Note that the films for the cyclin E immunoprecipitates (lower set) were exposed for 8 h to detect activity due to the low abundance of cyclin E in our cells, whereas the cyclin A films were exposed for 5 min.

al., 1994; Nourse, *et al.*, 1994; Polyak *et al.*, 1994a,b; Toyoshima and Hunter, 1994; Poon *et al.*, 1994), we used anti-cyclin A, anti-Cdk2, and anti-$p27^{Kip1}$ antibodies to immunoprecipitate lysates from untreated and growth-arrested WEHI-231 cells. We found that anti-cyclin A and anti-Cdk2 precipitates contained a 27-kDa band that co-migrated with $p27^{Kip1}$ (Figure 5). That this band was indeed $p27^{Kip1}$ was established as follows. Anti-cyclin A and anti-p27 antibodies were used to immunoprecipitate complexes from untreated or anti-IgM–treated ^{35}S-labeled WEHI-231 cells. The precipitates were dissociated by boiling in SDS-buffer and re-precipitated with anti-cyclin A or anti-p27. As shown in Figure 6, the amount of cyclin A–associated p27 (identified in the second immunoprecipitate) increased after anti-IgM treatment, as predicted. Likewise, the amount of cyclin A protein associated with p27 also increased in anti-IgM–treated WEHI-231 cells. By densitometric analysis, we estimated that the total amount of p27 increased at least fourfold, similar to the increase in the amount of p27 associated with cyclin A. That is, 17% of the cyclin A was associated with p27 before treatment (Figure 6A, compare lanes 1 and 3) but increased to 90% after treatment with anti-IgM (Figure 6A, lanes 2 and 4); in control cells, the amount of p27 associated with cyclin A was 60% (Figure 6A, compare lanes 5 and 7) and was 70% after treatment (Figure 6A, lanes 6 and 8). Similar results were obtained by directly probing anti-cyclin A immunoprecipitates of WEHI-231 lysates with anti-p27 antibodies (Figure 6B). No change in the level of the p27-related Cdk inhibitor p21 was observed in anti-IgM–treated WEHI-231 cells (Ezhevsky and Scott, unpublished results).

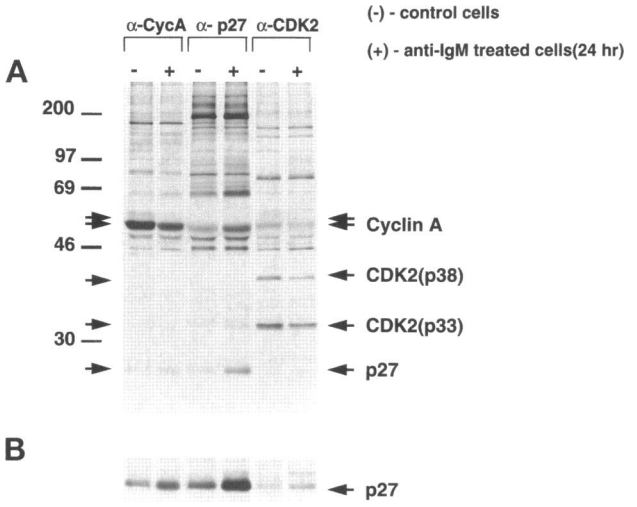

Figure 5. Immunoprecipitation of ^{35}S-labeled WEHI-231 cells. (A) Anti-IgM–treated (+) and untreated (−) B lymphoma cells were immunoprecipitated with anti-cyclin A, anti-p27, or anti-Cdk2, and electrophoresed. There was an increased association of p27 with cyclin A and Cdk2 in extracts from anti-IgM–treated cells (note arrows). (B) The same film was overexposed to better illustrate the changes in the bands in the 27-kDa range with anti-IgM.

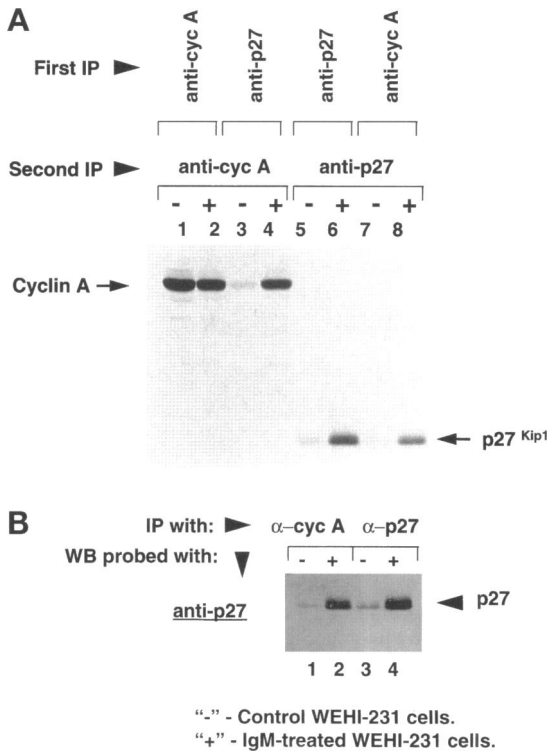

Figure 6. p27 is associated with the cyclin A kinase complex. (A) ^{35}S-labeled extracts from untreated and anti-IgM–treated WEHI-231 cells were precipitated for 16 h with anti-cyclin A or anti-p27 antibodies. The immunoprecipitates on protein A beads were boiled in SDS buffer, diluted up to 0.1% SDS, and then re-precipitated (second IP) with anti-cyclin A or anti-p27 and then electrophoresed. Densitometric comparison of lanes 1 and 3 indicated that the amount of cyclin A associated with p27 was 17% before treatment but was 90% after treatment with anti-IgM (lanes 2 and 4), whereas the amount of p27 associated with cyclin A was 60% in control cells (lanes 5 and 7) and 70% after treatment (lanes 6 and 8). (B) Effect on anti-IgM on $p27^{Kip1}$ levels in WEHI-231 lymphoma cells. Lysates from untreated (−) or anti-IgM treated (+, 24 h) WEHI-231 cells were immunoprecipitated with anti-cyclin A or anti-p27, and then electrophoresed and probed with anti-p27. The total amount of p27 was increased in growth-inhibited WEHI-231 cells in parallel with an increase in p27, which co-precipitated due to association with cyclin A.

Using the same approach, we immunoprecipitated cyclin A from WEHI-231 cell lysates treated with anti-IgM for different periods of time. The immunoprecipitates were electrophoresed, transferred to nitrocellulose, and then probed with anti-p27 antibodies. Additional aliquots were taken to measure the relative numbers of cells in G_1 by flow cytometry. We found that the amount of cyclin A–associated p27 correlated with the number of G_1-arrested cells during the course of anti-IgM treatment (Figure 7). Because the total amount of cyclin A-Cdk2 complexes did not increase in G_1-arrested WEHI-231 cells (see above), we can

assume that the p27/cyclin A ratio increased at least four- to fivefold, thus accounting for the inhibition of cyclin A–associated kinase activity.

Cdk4 Kinase Complexes Do Not Sequester p27^{Kip1}

Previous in vitro experiments have shown that p27^{Kip1} can inhibit cyclin A/E-Cdk2 and cyclin D-Cdk4 kinases, but that p27 preferentially binds to the cyclin D-Cdk4 complex (Toyoshima and Hunter, 1994). Therefore, one explanation for our results is that a decrease in cyclin D2 or Cdk4 expression might result in the "release" of p27 inhibitor that is then free to bind to cyclin A-Cdk2 complexes and block their activity. However, this is unlikely because the total amount of Cdk4 and cyclin D2 remained the same after anti-IgM treatment (Figure 8; Scott *et al.*, 1993). Nonetheless, to test directly for the association of p27 and Cdk4 in WEHI-231 cells, we performed anti-Cdk4 immunoprecipitation followed by immunoblot analyses (Figure 9A). These studies demonstrated that only a small portion of p27 was associated with Cdk4 in untreated cells. To exclude the possibility that the lower association of p27 with Cdk4 is due to reduced expression of Cdk4, we directly compared the fibroblast cell line 3T6 with WEHI-231 lymphoma cells. Immunoprecipitation with anti-cyclin D1, Cdk4, and p27 antibodies followed by immunoblotting with anti-Cdk4 antibodies revealed that relative levels of Cdk4 expression were the same in both tested cell lines (Figure 9B). However, the association of p27 with

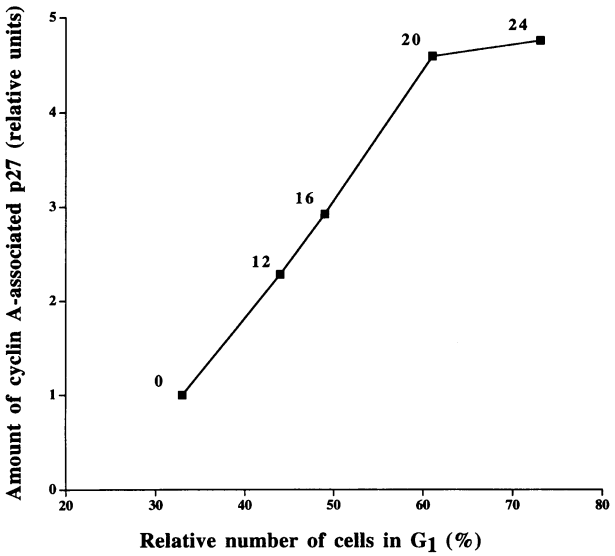

Figure 7. Kinetics of accumulation of p27 during growth inhibition of WEHI-231 lymphoma cells. At the indicated time points (in h), cell lysates were precipitated with anti-cyclin A and probed with anti-p27 as described in Figure 6B. Flow cytometric analysis of the percentage of cells in G1 was calculated as in Figure 1.

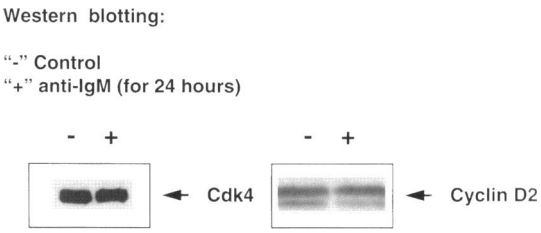

Figure 8. The effect of anti-IgM growth inhibition of WEHI-231 cells on Cdk4 and cyclin D2. Exponentially growing WEHI-231 cells were cultured alone (−) or with anti-IgM (+). Extracts were electrophoresed as described (see MATERIALS AND METHODS) and probed with anti-Cdk4 or anti-cyclin D2 antibodies. WEHI-231 cells treated with anti-IgM for 24 h showed no change in Cdk4 or cyclin D2 expression.

Cdk4 was readily detected in fibroblasts but not in the WEHI-231 B lymphoma. Hence, we suggest that the preferential binding of p27 to cyclin A may be a unique characteristic of B-lineage cells, and that Cdk4-cyclin D sequestration and release of p27 is not responsible for anti-IgM–mediated growth arrest.

P27-dependent Inhibition of Cyclin A–associated Kinase Activity Is Not Caused by Dissociation of Cyclin A-Cdk2 Complexes

We have shown that G1-arrest of WEHI-231 cells is not accompanied by an alteration in the total level of Cdk2 expression. Nonetheless, it is possible that the binding

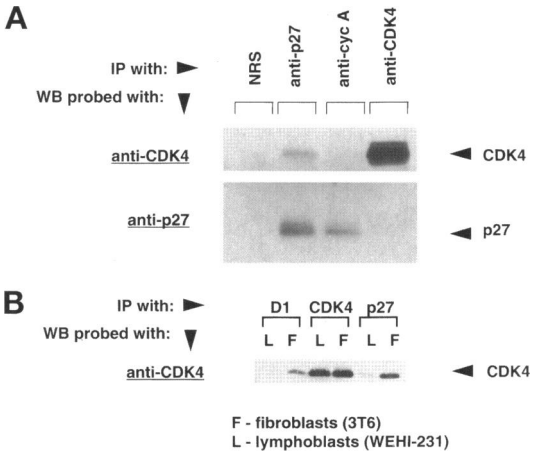

Figure 9. Association of p27 with cyclin A and Cdk4 in WEHI-231 cells. (A) Untreated WEHI-231 cell lysates were immunoprecipitated (IP) with anti-p27, anti-cyclin A, or anti-Cdk4. The precipitates were electrophoresed and then probed (WB) with anti-Cdk4 or anti-p27 antibodies. (B) Lysates of WEHI-231 lymphoma cells (L) or 3T6 fibroblasts (F) were immunoprecipitated with anti-cyclin D1, anti-Cdk4, or anti-p27 antibodies, and then probed with anti-Cdk4 antibodies. Equivalent amounts of Cdk4 are found in both cell types, but p27 association with Cdk4 is minimal in the lymphomas; cyclin D1 is absent from the WEHI-231 lymphoma cells.

of p27 to cyclin A or some other unrelated event might cause the dissociation of cyclin A-Cdk2 complex or prevent the phosphorylation of Cdk2 on threonine 160 by a Cdk-activating kinase. To test this, we immunoprecipitated the cyclin A and p27 complexes from cell lysates at different time points after the addition of anti-IgM and probed the precipitates with anti-Cdk2 antibodies (Figure 10). The cyclin A immunoprecipitates contained the 33- and 38-kDa forms of Cdk2. Anti-IgM treatment did not decrease the level of either Cdk2 form associated with cyclin A; thus, the level of cyclin A-Cdk2 complex was unchanged despite the loss of cyclin A–associated kinase activity and G_1 arrest. In contrast, the amount of both Cdk2 proteins in p27 immunoprecipitates increased during the course of G_1 arrest as reflected by the increase in the level of cyclin A-Cdk2 complex associated with p27 (Figure 10). Indeed, probing of cyclin A immunoprecipitates with anti-p27 antibodies confirmed this conclusion: the relative amount of p27 bound to cyclin A complexes increased in parallel with cell accumulation in G_1 phase (Figure 7). In conclusion, anti-IgM–mediated growth arrest of WEHI-231 cells inhibits cyclin A-Cdk2 complex activity primarily by increasing their p27 content.

DISCUSSION

Cross-linking of surface IgM on B-lymphoma cells in early G_1 prevents pRb phosphorylation and results in growth arrest before entry into S phase. In contrast, by late G_1, when pRb is primarily in the hyperphosphorylated state, anti-IgM affects neither pRb phosphorylation nor cell cycle progression. Thus, the early G_1 dependence of anti-IgM inhibitory activity reflects the state of phosphorylation of pRb and is a critical decision point for growth arrest versus progression (Joseph *et al.*, 1995). To explore the basis for the effects of anti-IgM on pRb phosphorylation further, we decided to search for the cyclin-Cdk complex that could phosphorylate this growth suppressor in vitro, and which is modulated by anti-IgM treatment with the same kinetics. Treatment of unsynchronized WEHI-231 with anti-IgM inhibits the cyclin A–associated kinase activity in parallel with cell cycle arrest in late G_1 (Joseph *et al.*, 1995). We show herein that formation of an active cyclin A-Cdk2 kinase complex is inhibited by anti-IgM treatment of cells in early G_1. However, once active complexes have been formed in late G_1, anti-IgM treatment is ineffective. Our studies have established that one of the specific targets of anti-IgM treatment is the cyclin A-Cdk2 complex, which is inhibited by increased levels of the $p27^{Kip1}$ Cdk inhibitor when WEHI-231 cells are treated with anti-IgM in early G_1.

In anti-IgM–arrested WEHI-231 B lymphoma cells there is no apparent decrease in the level of cyclin A-Cdk2 complexes, but nearly all the cyclin A is associated with p27, a result which is consistent with p27 being responsible for the observed inhibition of cyclin A-Cdk2 activity. Conversely, nearly all of the p27 is bound to cyclin A-Cdk2 complexes, which explains why there is no free Cdk inhibitor activity in anti-IgM–treated cell lysates. Previously, we reported that cyclin A activity remains the same up to 12 h of treatment and drops between 12 and 24 h (Joseph *et al.*, 1995). Although we have not performed detailed kinetic experiments to compare the level of cyclin A-bound p27 with the activity of cyclin A–associated kinase, our results suggest that the amount of p27 accumulating in the cyclin A complex increases gradually (Figure 7). The simplest explanation for the lag in inhibition of cyclin A-Cdk2 activity is that a saturating amount of p27 is needed to achieve kinase inhibition, as is the case for the related p21 inhibitor of cyclin A-Cdk2 (Zhang *et al.*, 1994; Harper *et al.*, 1995). In transforming growth factor (TGF)-β–treated Mv1Lu cells a fourfold increase in the level of p27 bound to cyclin E-Cdk2 is sufficient to cause complete inhibition (Reynisdottir *et al.*, 1995). By analogy, the four- to fivefold increase in cyclin A-Cdk2–associated p27 that we found in anti-IgM–treated WEHI-231 cells is likely to be sufficient to account for the observed inhibition of cyclin A-Cdk2 activity. We propose that anti-IgM treatment of early G_1 WEHI-231 B-lymphoma cells

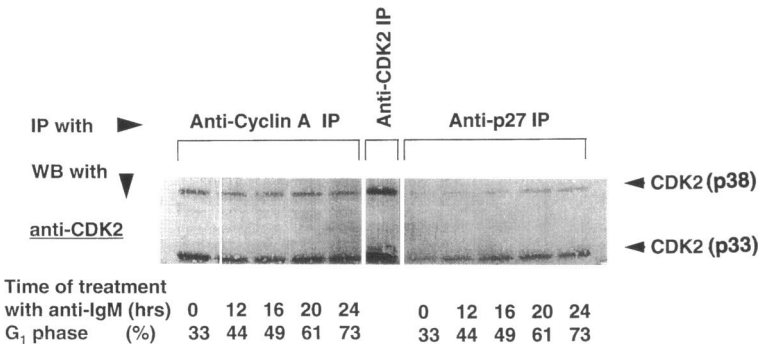

Figure 10. Accumulation of p27 in cyclin A complexes does not disrupt cyclin A-Cdk2 association. WEHI-231 cells were cultured with anti-IgM as in Figure 7 and lysates immunoprecipitated with anti-cyclin A or anti-p27, and probed with anti-Cdk2. Although the amount of Cdk2 associated with cyclin A was similar at all time points, the amount of Cdk2-associated p27 increased with time of anti-IgM treatment, in parallel with increases in p27 (our unpublished results).

increases the level of p27 above that needed to cause complete inactivation of cyclin A-Cdk2 complexes, which limits the phosphorylation of pRb and, thus, causes growth arrest.

Our data show that inhibition of cyclin A–associated kinase activity by anti-IgM treatment in early G_1 correlates with an increase in the amount of the p27 in the cyclin A complex. The precise mechanism of p27 inhibition of cyclin-Cdk kinase activity has not been defined, although it is known that the N-terminal inhibitory domain interacts with both the cyclin and Cdk subunits. In the case of the related inhibitor p21, there is evidence that two molecules of p21 are required to inhibit a single cyclin-Cdk complex (Zhang *et al.*, 1994). It is not yet known whether more than one p27 molecule is required for cyclin-Cdk inhibition, but the fact that H1 kinase activity can be detected in anti-p27 immunoprecipitates from growing cells suggests that this may be the case. We do not know the exact stoichiometry of p27 bound to cyclin A-Cdk2 in anti-IgM–treated cells, but one can assume that when there is an excess of p27, the cyclin-Cdk complexes will be in an inhibited state. Consistent with this view, we show that the level of cyclin A–associated kinase activity depends on the p27/cyclin A ratio, and that anti-IgM treatment increases that ratio concomitant with a decrease in cyclin A-Cdk2 activity.

Why does treatment of early G_1 cells cause such an effect, whereas treatment of late G_1 cells does not? The mechanism(s) leading to increased p27 expression (eg. transcription and protein stabilization) and the exact kinetics of its increase are not known at present, but we suggest that the p27 levels rise relatively slowly in response to anti-IgM treatment, and that only when they start to rise in early G_1 does the level of p27 become great enough to inhibit all the cyclin A-Cdk2 complexes that are formed as cyclin A levels rise in late G1. The increase in p27 in early G_1 may also counteract a normal drop in cyclin A–associated p27 levels that occurs when cells move from early to late G_1, which is required for entry into S phase, as has been noted in the case of Rat-1 cells (Resnitzky *et al.*, 1995). In contrast, when cells are treated in late G_1, p27 may not reach a high enough level to inhibit cyclin A-Cdk2 before the cells have committed to enter S phase. It is also formally possible that anti-IgM does not lead to increased p27 during late G_1 and S. Although this seems unlikely, further experiments are needed to resolve this issue.

Rabbit anti-cyclin A antiserum recognized two bands in immunoblots and the same two forms of cyclin A could be precipitated from ^{35}S-labeled WEHI-231 cell lysates. The faster migrating form of cyclin A is common to both human and murine cells and corresponds to that originally described as p58–60 kDa human cyclin A protein (Ezhevsky and Scott, unpublished results; Bui *et al.*, 1993; Carbonaro-Hall *et al.*, 1993). Anti-IgM did not dramatically change the abundance of this form in unsynchronized cells, even after 24 h, by which time virtually all cells have been arrested in late G_1. Moreover, the level of expression of this form of cyclin A did not change dramatically upon incubation of cells either from early or late G_1 fractions with anti-IgM. In contrast, the minor, slower migrating form of cyclin A disappeared as a result of anti-IgM treatment in unsynchronized WEHI-231 cells, concomitantly with the loss of cyclin A-Cdk2 activity, whereas surface IgM cross-linking had no effect on the level of the slower migrating form in late G_1 cells that have already expressed this form of cyclin A and have high cyclin A-Cdk2 activity. This suggests, but does not prove, that the appearance of the slower migrating form reflects cyclin A–associated kinase activity. Similar observations have recently been made for cyclin E; the level of cyclin E–associated kinase activity is correlated with the level of expression of a slower migrating form rather than with the total levels of cyclin E protein (Dulic *et al.*, 1994). Treatment with potato acid phosphatase increased the mobility of this slower form of cyclin E, but we found that this treatment did not affect either form of cyclin A in these complexes, implying that the upper form of cyclin A is not generated by phosphorylation (our unpublished results). Due to the low abundance of cyclin E protein in WEHI-231 cells, we could not determine whether cyclin E–associated kinase activity correlated with a slower migrating form of cyclin E, but we were able to show that anti-IgM treatment led to a decrease in cyclin E–associated kinase activity (Figure 4).

Rabbit anti-Cdk2 antiserum recognized 38- and 33-kDa bands on immunoblots of lysates from WEHI-231 cells. The 38-kDa Cdk2 form is presumably a murine-specific form of Cdk2 generated by alternative splicing (Yasuda *et al.*, 1993), although the function of this form is unknown. The 33-kDa band corresponds to the major form of Cdk2 found in all mammals. This form is often resolved into a doublet when exponentially growing human and murine cells are analyzed (Pagano *et al.*, 1993; Tsai *et al.*, 1993; Tanguay and Chiles, 1994). The faster migrating form, resulting from phosphorylation of threonine 160 by a Cdk-activating kinase in other cell types (Darbon *et al.*, 1994), is associated with dramatically increased Cdk-dependent kinase activity (Connell-Crowley *et al.*, 1993). Indeed, the appearance of the faster migrating form of Cdk2 usually coincides with entrance into S in fibroblasts (Koff *et al.*, 1993; Pagano *et al.*, 1993; Tsai *et al.*, 1993). Phosphorylation of threonine 160 can be inhibited by p27 in vitro (Kato *et al.*, 1994; Polyak *et al.*, 1994a; Aprelikova *et al.*, 1995). Inhibition of threonine 160 phosphorylation is also observed in TGF-β–treated Mv1Lu mink lung epithelial cells where accumulation of p27 bound to cyclin E-Cdk2 complexes results in a decrease in the level of the threonine 160 phosphory-

lated form of Cdk2 (Koff *et al.*, 1993; Reynisdottir *et al.*, 1995). Unfortunately, the 33-kDa Cdk2 band was not consistently resolved into a doublet in our experiments with WEHI-231 B-lymphoma cells, and therefore we cannot determine whether there was decreased phosphorylation of threonine 160 in anti-IgM–treated cells, although this seems likely to be the case. Such inhibition of threonine 160 phosphorylation may contribute significantly to diminished cyclin A-Cdk2 activity in addition to the direct inhibition of activity exerted by bound p27.

The block of the G_1 to S transition in the WEHI-231 B-cell lymphoma model has similarities and differences to the G_1 block imposed by TGF-β on cell lines sensitive to this cytokine. Like TGF-β, the action of anti-IgM does not appear to be at the level of expression of cyclins and Cdks or their association, because the levels of Cdk4, Cdk2, cyclin D2, and cyclin A, and the association of cyclin A with Cdk2 are unaffected by anti-IgM (see Figures 8 and 10; Scott *et al.*, 1995). Moreover, in both cases the G_1 block appears to be a result of the accumulation of inactive cyclin-Cdk2 complexes containing bound p27. However, the mechanisms through which this is achieved are different.

TGF-β–arrested epithelial cells show a rapid increase in the level of p15, a $p16^{INK4}$ family Cdk inhibitor, which leads to displacement of p27 from cyclin D-Cdk4/Cdk6 complexes, and subsequent binding of p27 to cyclin E-Cdk2 complexes, which accumulate in an inactive state containing only the form of Cdk2 lacking phosphate at threonine 160 (Polyak *et al.*, 1994a; Hannon and Beach, 1994; Reynisdottir *et al.*, 1995). We do not know whether anti-IgM treatment increases expression of p16 family Cdk inhibitors in WEHI-231 cells, but the fact that there is no effect of anti-IgM on cyclin A expression, which normally requires cyclin D function, implies that cyclin D-Cdk4/6 complexes are active. Moreover, very little p27 is bound to cyclin D-Cdk4 complexes in WEHI-231 cells, precluding p16 family-induced redistribution of p27 as a mechanism of inhibiting cyclin-Cdk2 complexes. Long-term treatment of Mv1Lu mink lung epithelial cells with TGF-β also results in reduced levels of Cdk4 and Cdk2, which may contribute to the induction of a long term quiescent state (Ewen *et al.*, 1993; Reynisdottir *et al.*, 1995). We did not observe any alteration in cyclin D or Cdk4 level induced by anti-IgM or even TGF-β in the WEHI-231 or CH31 B lymphomas by immunoblotting (our unpublished observations; Figure 9). However, in a recent report, Ishida and colleagues (1995) found that Cdk4 was reduced in growth-arrested anti-IgM–treated WEHI-231 cells. This discrepancy may be explained by the kinetics of growth arrest in different WEHI-231 clones, because prolonged arrest can lead to decreases in the amounts of these Cdks in other cells types (Reynisdottir *et al.* 1995).

The major difference in the mechanism of G_1 arrest is that anti-IgM treatment increases the levels of p27 in WEHI-231 cells, whereas TGF-β does not change the overall levels of p27 in Mv1Lu cells (or in WEHI-231 cells; our unpublished observations). Thus, although both anti-IgM and TGF-β cause G1 arrest through the accumulation of the inactive p27-bound cyclin-Cdk2 complexes, this is achieved in different manners; in anti-IgM–treated cells it is due to an increase p27 whereas in TGF-β–treated cells it is a result of redistribution of existing p27. These differences are in agreement with our previous conclusion that a TGF-β–independent pathway may be responsible for the pRb underphosphorylation and cell cycle blockade in these murine B-cell lymphomas (Warner *et al.*, 1992). Thus, a number of conclusions regarding the effect of TGF-β on cell cycle progression in epithelial cells may not reflect growth arrest events seen in other cell lineages, like lymphocytes. This is an important caveat in generalizations regarding cell cycle restriction points.

As discussed above, p27 has been implicated in negative regulation of cell growth, presumably by binding to and preventing activation of cyclin-Cdk complexes and by inhibiting already activated complexes (Firpo *et al.*, 1994; Kato *et al.*, 1994; Nourse *et al.*, 1994; Polyak *et al.*, 1994a; Toyoshima and Hunter, 1994). In our experiments, we found that p27 is preferentially associated with cyclin A-Cdk2, rather than with cyclin D-Cdk4, even though the amounts of cyclin D and Cdk4 are similar in WEHI-231 cells and murine fibroblasts. Moreover, p27 binds to the cyclin A-Cdk2 complexes even in the absence of anti-IgM treatment. In mouse fibroblasts, p27 is preferentially bound to cyclin D-Cdk4/Cdk6 complexes in G_1, and is released as cells progress into S phase, apparently as a result of dissociation of p27 in S phase, even though cyclin D and Cdk4 levels remain high (Poon *et al.*, 1995). This S phase phenotype is similar to the situation we have observed in WEHI-231 cells.

The mechanism through which anti-IgM treatment increases p27 levels in WEHI-231 cells is not known. A similar increase in p27 levels has been reported in cyclin AMP-induced growth arrest in macrophages, but the mechanism underlying the increase was not established (Kato *et al.*, 1994). It will be important to determine whether anti-IgM treatment increases p27 mRNA levels, because Pagano *et al.* (1995) have shown that the increase in p27 level in quiescent mouse fibroblasts is due to a decreased rate of turnover. Thus, the increase in WEHI-231 cells could be due to protein stabilization rather than increased synthesis. Experiments are in progress to examine how anti-IgM regulates the synthesis, degradation, or availability p27 and its relationship with the activation of the cyclin A-Cdk2 complex in these B-lymphoma cells.

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