Modulation of E2F Activity via Signaling through Surface IgM and CD40 Receptors in WEHI-231 B Lymphoma Cells*

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Stimulation of the phenotypically immature B cell lymphoma WEHI-231 with anti-IgM induces G₁ arrest followed by apoptotic cell death, which can be reversed by stimulation via the CD40 receptor. Here, we show that cells expressing bcl-x₅ (WEHI-bcl-x₅) arrest at G₀/G₁ following culture with anti-IgM but do not undergo apoptosis. These arrested cells can be induced to reenter the cell cycle by ligation of CD40. We have therefore used these cells as a model to study the regulation of the transcription factor E2F, which is critically involved in transit through the cell cycle. We found that anti-IgM treatment induces the appearance of an inhibitory DNA binding complex containing the pRB-related pocket protein p130, thereby releasing the repression of E2F, which is critically involved in the cell cycle. We subsequently found that these arrested cells can be induced to reenter the cell cycle by ligation of CD40. These effects were reversed following stimulation via CD40. These changes in free E2F levels were regulated by changes in E2F1 gene transcription, which is at least partly a result of control of E2F1 promoter activity through its E2F binding sites. Transient transfection experiments showed that either E2F1 or the viral oncoprotein E1A, which sequesters pocket proteins, including p130, overcame anti-IgM-induced cell cycle arrest in WEHI-bcl-x₅. Taken together, these results indicate that in WEHI-231 sIgM ligation induces the accumulation of hypophosphorylated p130 with consequent inhibition of E2F1 gene transcription and cell cycle arrest. Conversely, ligation of CD40 causes hyperphosphorylation of p130, thereby releasing the repression of E2F1 and other E2F-regulated genes, enabling the cells to reenter the cycle. These results, therefore, provide novel insights into the mechanisms whereby antigen receptors on immature B cells deliver inhibitory signals (leading to negative selection of self-reactive B cells) and how these signals can be modulated by positive signals generated via CD40.

A key mechanism for the control of autoreactivity within the immune system is the purging of self-reactive clones during the early stages of T and B lymphocyte development (1–3). In B lymphocytes, such negative selection occurs in immature B cells within the bone marrow or in recent bone marrow emigrants. Studies with a variety of transgenic strains of mice have clearly shown that encounter of immature B lymphocytes with self antigen can lead to growth arrest followed by apoptotic cell death (clonal deletion) or to functional silencing (clonal anergy), depending on the degree of antigen receptor cross-linking (4–8). Currently, little is known about the intra- and extracellular events that lead to deletion or anergy or, indeed, how negative selection can be modulated by co-stimulatory (e.g., T cell-derived) signals.

A well established in vitro model for studying clonal deletion of B cells is the WEHI-231 B cell lymphoma. These phenotypically immature (sIgMᵇ⁻sIgDᵇ⁻) cells arrest in the G₀ phase of the cell cycle following culture with anti-IgM antibodies and subsequently die by apoptosis (9–11). Significantly, these events can be abrogated by concurrent stimulation via the CD40 receptor (12). CD40 is now known to play a central role in the initiation of T cell-dependent antibody responses, via its interaction with a counterreceptor, the CD40-ligand, which is expressed on activated CD4 T helper cells. CD40-mediated signals in normal B cells promote cellular proliferation, immunoglobulin isotype switching, germinal center formation, and the development of B memory cells (13, 14). In addition, CD40 appears to be the most important receptor on B cells for promoting cell survival and in reversing sIg-induced apoptotic signals (15–17).

We and others have found that CD40 stimulation rapidly induces the appearance of the anti-apoptotic protein bcl-x₅ in WEHI-231 cells (17–20). However, although constitutive expression of bcl-x₅ protects these cells from anti-IgM-induced apoptosis, it fails to reverse growth arrest, so that anti-IgM-treated cells become arrested in the G₀/G₁ phase of the cell cycle. We subsequently found that these arrested cells can be induced to reenter the cell cycle by stimulation with anti-CD40 (in the continuing presence of anti-IgM). In this work, we used bcl-x₅-transfected WEHI-231 cells (WEHI-bcl-x₅) to study how negative signals via sIgM and positive ones via CD40 control the activity of E2F, a family of transcription factors that play key roles in cell cycle progression.

The transition from G₁ to S is an important regulatory point in cell cycle progression and E2F plays a central role at this checkpoint by regulating the transcription of a number of genes required for DNA synthesis and cell cycle regulation (22, 23), including the E2F1 gene itself (24–26). There is accumulating evidence that E2F binding sites can act as either positive (activating) or repressive elements in controlling these cell cycle regulatory genes (27, 28). E2F is a generic name given to a group of heterodimeric transcription factors, each consisting of one member of the E2F family of proteins (E2F1–5), together with one member of the DP family (DP1–3) (28–32). These
proteins interact in different combinations to generate distinct E2F activities. E2F activity is negatively regulated by the so-called pocket proteins (pRB, p107, and p130). It also appears that individual E2F protein associates preferentially with a particular member of the pocket protein family in vivo. Thus, E2F1, E2F2, and E2F3 interact specifically in vivo with pRB (33). E2F4 binds preferentially to p107 and p130 (34, 35), whereas E2F5 complexes only with p130, under normal physiological conditions (29, 30).

These pocket proteins interact with E2F at distinct stages of the cell cycle (36). Hence, p130/E2F2 complexes are found predominantly at G0/early G1, p107/E2F complexes are found in G1 and S, and pRB/E2F complexes are found in late G1 and S. These temporal interactions are governed by the phosphorylation status as well as by the expression patterns of the pocket proteins. These proteins are major targets of the cyclin-dependent kinases, in association with their regulatory partners the cyclins. Hypophosphorylated pRB is first detected in early G1 and becomes hyperphosphorylated during late G1 and S (37–39). Hypophosphorylated p107 is present at low levels in G0 and increases dramatically during G1, with accompanying hyperphosphorylation (40). Hypophosphorylated p130 is found in quiescent (G0) cells and early in G1; in mid-G1 the protein becomes hyperphosphorylated, and this form persists throughout the remainder of the cycle (41). Hyperphosphorylation of p130 in mid-G1 plays a major role in the relieving E2F-mediated repression of G0/S genes, especially of the E2F1 gene itself (42). Overexpression of E2F1 activity has previously been shown to be able to induce cells to enter S phase of the cell cycle; however, these cells demonstrate an increase in apoptosis (43–46). Because E2F is the converging point for integrating positive and negative signals within the cell cycle, it was an attractive possibility that its activity would be regulated in response to sIgM or CD40-mediated signals in WEHI-231. The results of the experiments reported here provide evidence for this concept.

EXPERIMENTAL PROCEDURES

Tissue Culture and Anti-IgM and Anti-CD40 Treatment of WEHI 231 bcl-xL Cells—Wild-type WEHI 231 cells, which express bcl-2 but no detectable bcl-xL, undergo apoptotic cell death when cultured with anti-IgM. For these experiments, we therefore used WEHI 231 bcl-xL cells, which were stably transfected with bcl-xL (kindly provided by Dr. C. Thompson). These cells express bcl-xL at high levels and do not undergo apoptosis following treatment with anti-IgM (18). They were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin/streptomycin, and 50 µM 2-mercaptoethanol. To induce growth arrest, the cells were cultured for the indicated periods of time with 1 µg/ml of a monoclonal rat anti-IgM (clone b.7.6; Ref. 18). The cells were induced to reenter the cell cycle by treatment of WEHI 231 expressing bcl-xL (WEHI-bcl-xL), which do not undergo apoptosis following treatment with anti-IgM (18). Instead these cells gradually accumulated at G0/G1 phase of the cell cycle, with a concomitant reduction of cells in S and G2/M (Fig. 1). For these experiments, we therefore used WEHI 231 bcl-xL cells, which were stably transfected with bcl-xL (kindly provided by Dr. C. Thompson). These cells express bcl-xL at high levels and do not undergo apoptosis following treatment with anti-IgM (18). They were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin/streptomycin, and 50 µM 2-mercaptoethanol. To induce growth arrest, the cells were cultured for the indicated periods of time with 1 µg/ml of a monoclonal rat anti-IgM (clone b.7.6; Ref. 18). The cells were induced to reenter the cell cycle by subsequent stimulation with a rat anti-CD40 monoclonal antibody (3/ 23; Ref. 11) at 10 µg/ml.

Transfections and Reporter Assays—The mouse wild-type E2F1 promoter-luciferase reporter construct, pE2F1(176/+36)-Luc, and the mutant derivative with E2F sites mutated, pE2F1(176/+36ΔΔ)-Luc, have been described previously (24). 106 WEHI 231 cells were electroporated with 10 µg of luciferase reporters and 2 µg of pSV-b-gal (Promega) at 250 V and 960 microfarads. Eighteen h after transfections, the cells were harvested and lysed in cell culture lysis reagent (Promega) at room temperature for 10 min. The extracts were then cleared by centrifugation and assayed for luciferase and β-galactosidase activities as described previously (28). The relative promoter activity was calculated from the ratio of the luciferase to β-galactosidase activities.

For flow cytometric analyses, WEHI 231 cells were transfected with 10 µg of E1A or E2F1 expression vector and 2 µg of phCD8 (47) using electroporation in medium as described above. The expression vectors for wild-type and mutant E1A (48) or E2F1 (43) have been described previously.

Flow Cytometric Analyses—Cell cycle analysis was performed by flow cytometry using propidium iodide as described previously (28). Cells were collected by centrifugation and washed with PBS before being fixed in 90% ethanol and 10% PBS prior to DNA staining. When appropriate, the cells were incubated with 1 µg/ml fluorescein isothiocyanate-conjugated anti-CD8 antibody (Dako A/S, Denmark) in PBS with 5% FCS for 30 min at 4 °C before fixing with ethanol. Cells were then stained with propidium iodide (20 µg/ml) in the presence of 10 µg/ml RNase A (Sigma) and stored at 37 °C for 30 min prior to analysis on a Becton Dickinson FACSort flow cytometer. The cell cycle profile was analyzed using the Cell Quest software.

Gel Retardation and Super-shift Assays—Whole cell extracts were prepared as described previously (49). Protein yield was quantified by Bradford analysis (Bio-Rad). E2F gel retardation assays were performed essentially as described (28), using a double stranded oligonucleotide containing the distal E2F2 binding site from the adenovirus type 5 E2a promoter (49). Twenty µg of whole cell extract was incubated with 1–2 ng of 32P-labeled DNA probe in the presence of 2 µg of sonicated salmon sperm DNA and 200 ng of a comparable unlabeled double stranded oligonucleotide with a mutated E2F site at 30 °C for 15 min. The reactions were electrophoresed on 4% polyacrylamide gels in 0.25× TBE buffer. The gels were then dried and exposed to x-ray films. Super-shift assays were performed by adding 1–2 µl of concentrated antibodies to the gel shift reaction prior to the addition of probe.

Rabbit anti-pRB/p130 and anti-cyclin A antisera were kindly provided by Drs. A. Giordano and J. Pines, respectively. Anti-pRB mouse monoclonal antibody 21C9 (50) was a generous gift from Dr. Sybille Mittnacht. Anti-DP1, E2F1, E2F4 rabbit antisera were raised against synthetic peptides corresponding to unique carboxy-terminal regions (amino acids 410–418, 435–457, and 397–413) of their respective human proteins.

Western Blot Analyses—Fifty µg of whole cell extract was separated on SDS-polyacrylamide gels. Following electrophoresis, proteins were transferred to Immobilon-P polycylinide difluoride membrane (Millipore), detected with specific antibodies, and visualized by the ECL detection system (Amersham Pharmacia Biotech). The anti-DP1 monoclonal antibody was raised against the amino-terminal region of the human DP1 protein. Rabbit polyclonal antibodies against E2F1C-20, E2F4C-108, p107(C-18), pRB(C-15), and p130(C-20) were purchased from Santa Cruz Biotechnology.

RNase Protection Assay—Total RNA was isolated using the RNAzol reagent (Biogenex) and quantified by absorbance at 260 nm. RNase protection assays were performed using the RPAII kit (Ambion). The RNA probe was synthesized from a plasmid pCDNA3.E2F1x containing a mouse E2F1 DNA (51) (kindly provided by Dr. Peggy Farnham) (nucleotides 1–1340) fragments cloned into the pCDNA3 expression vector (Invitrogen). The plasmid was linearized with BamHI to give a template for synthesis of a 211 nucleotide RNA probe (+1129 to +1340) using SP6 RNA polymerase in the presence of [32P]UTP. The control 18S ribosomal RNA probe was prepared from the vector pT7 RNA 18S (Ambion) using T7 RNA polymerase. The protected RNA fragments were resolved using 6% acrylamide gels.

RESULTS

Cross-linking of sIgM on WEHI 231 Expressing bcl-xL Induces G0/G1 Arrest, Which Is Overcome by CD40 Signaling—Because treatment of WEHI-231 with anti-IgM induces apoptosis, it is not feasible to study the long term effects of signaling via sIgM independently of the long term effects of CD40 in these cells. To circumvent this problem, we used WEHI-231 cells stably expressing bcl-xL (WEHI-bcl-xL), which do not undergo apoptosis following culture with anti-IgM (18). Instead these cells gradually accumulated at G0/G1 phase of the cell cycle, with a concomitant reduction of cells in S and G2/M (Fig. 1). By 24 h, over 80% of the cells were arrested in G0/G1, and nearly all of the cells were arrested at the G0/G1 phase of the cell cycle after 48 h of treatment with anti-IgM. Notably, none of the WEHI bcl-xL cells underwent apoptosis following culture with anti-IgM (18). Instead these cells gradually accumulated at G0/G1 phase of the cell cycle, with a concomitant reduction of cells in S and G2/M (Fig. 1). By 24 h, over 80% of the cells were arrested in G0/G1, and nearly all of the cells were arrested at the G0/G1 phase of the cell cycle after 48 h of treatment with anti-IgM. Notably, none of the WEHI bcl-xL cells underwent apoptosis following culture with anti-IgM (18). Instead these cells gradually accumulated at G0/G1 phase of the cell cycle, with a concomitant reduction of cells in S and G2/M (Fig. 1).
Stimulation via sIgM or CD40 Induces Changes in E2F DNA Binding Complexes—To determine whether stimulation of these cells via sIgM or CD40 affects E2F activity, we initially performed gel mobility shift experiments on extracts from WEHI-bcl-xL, using an E2F-specific oligonucleotide. Anti-IgM and anti-CD40 treatment elicited substantial quantitative changes in the faster migrating E2F complexes (complex A), which were previously shown to consist of “free” E2F (52, 28), and the slower mobility complexes (complexes B and C), which are known to contain the pRB family of related proteins (Fig. 2A). To identify the components of these DNA binding activities, we therefore performed super-shift assays (Fig. 2B), using specific antibodies against different components of E2F complexes, on cycling (anti-IgM, 0 h) as well as on anti-IgM arrested WEHI bcl-x₁ cells (anti-IgM, 48 h). These experiments showed that the faster migrating free E2F complex contained mainly E2F1 and DP1. The levels of this free E2F activity declined after anti-IgM treatment, becoming almost undetectable after 24 h, and reappeared very rapidly after anti-CD40 treatment, reaching a peak at 12 h. The accumulation of this free DP1/E2F1 is associated with cells in late G1 and S phases. Overexpression of this free E2F1/DP1 activity has previously been shown to drive cells into S phase (53). Consequently, it is likely that the accumulation of this free E2F1/DP1 complex is functionally important in driving cells to reenter the cell cycle in response to CD40 stimulation. The slowest migrating complex (complex C), which contained p107, E2F4, DP1, and cyclin A, was detected in untreated WEHI-bcl-x₁ cells and in arrested cells 12 h after anti-CD40 treatment (Fig. 2A). This complex is similar to the S phase-specific complex previously found in T-lymphocytes and fibroblasts (54, 55, 28) and is believed not to be repressive to E2F activity. It has been suggested that the p107/cyclin A-containing E2F complex could mediate the phosphorylation of E2F/DP heterodimers and thus have a role in regulating E2F transcriptional activity (40). The other, slower migrating species (complex B) consisted of E2F4/DP1 complexed with either p130 or p107. This complex accumulated progressively after anti-IgM treatment, reaching a peak at
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48 h, and disappeared after subsequent culture with anti-CD40, concomitant with cells reentering the cell cycle from G0/G1 (Fig. 1). Both the p130 and p107-E2F complexes have previously been shown to repress E2F-mediated transcription and correlate with G0/G1 arrest (28, 56–59). It is therefore conceivable that the p130 and p107/E2F complexes play an important role in mediating cell cycle arrest in response to sIgM signaling.

It is noteworthy that the level of free E2F is different between cells treated with anti-IgM for 48 h and those that were in anti-CD40 0 h. This is because the two parts of the experiments were performed with different batches of cells. The differences observed (also seen in Fig. 5) therefore presumably reflect the completeness of growth arrest, with consequent disappearance of free E2F.

Ligation of sIgM or CD40 Induces Changes in Expression of Components of E2F Complexes—To understand the molecular events underlying the changes in E2F DNA binding complexes in response to anti-IgM and anti-CD40 stimulation, we examined the expression patterns and phosphorylation status of E2F components and pocket proteins by Western blotting. The results showed that both pRB and p107 were present in cycling cells, but their levels decreased dramatically 48 h after treatment with anti-IgM (Fig. 3). In addition, both pRB and p107 became hypophosphorylated (Fig. 3, lower bands) after anti-IgM treatment. In contrast, p130 was almost undetectable in cycling WEHI-bcl-xL cells, and stimulation with anti-IgM induced an accumulation of p130 in its hypophosphorylated form (Fig. 3, lower bands). After treatment with anti-CD40, p130 rapidly became hyperphosphorylated (Fig. 3, upper band), and this was closely followed by accumulation of hyperphosphorylated pRB and p107 (Fig. 3, upper bands) at 12 h. These results therefore implied that p130 could be the primary effector of the negative proliferative signal mediated by sIgM.

Further Western blot analyses also indicated that E2F1 was present in cycling WEHI bcl-xL (Fig. 4). Treatment with anti-IgM down-regulated the expression of E2F1 so that the protein was almost undetectable at 48 h. Cross-linking of CD40 reversed this effect, and maximal levels of E2F1 expression occurred 12 h post anti-CD40 stimulation. In contrast, the level of DP1 only declined slightly in response to anti-IgM treatment and recovered gradually after anti-CD40 treatment. The expression pattern of E2F1 mirrors the changes in level of the E2F1/DP1-containing free E2F complex in response to anti-IgM and anti-CD40 treatments, implying that the formation of the free E2F1/DP1 complex is rate limited by the expression of E2F1.

Kinetics of E2F1 mRNA Level Reflect That of the E2F1 Protein following Anti-IgM and Anti-CD40 Treatment—To investigate whether the changes in E2F1 protein levels in response to sIgM or CD40 stimulation is a result of alterations in E2F1 mRNA transcript levels, we performed an RNase protection assay, using a probe corresponding to a carboxyl-terminal coding region of the murine E2F1 cDNA (Fig. 5). This showed that, like the E2F1 protein level, the E2F1 mRNA transcripts declined progressively after the cross-linking of sIgM, reaching a minimum at 48 h posttreatment and increased rapidly after anti-CD40 treatment. Levels of control 18S ribosomal RNA level remained constant throughout the experiment. Hence, the kinetics of E2F1 mRNA accumulation mirror that of the E2F1 protein after anti-IgM and anti-CD40 treatments (Fig. 4), suggesting that modulation of E2F1 levels in response to these stimuli is predominantly regulated at the level of transcription. Again, the low residual level of E2F1 mRNA in the anti-CD40 0 h sample is due to batch to batch variation of cellular growth arrest induced by anti-IgM.

Signaling via sIgM and CD40 Is Mediated through the E2F Sites of the E2F1 Gene—The transcriptional activity of the E2F1 gene following anti-IgM and anti-CD40 treatment was monitored using a mouse E2F1 promoter/luciferase reporter construct, which was transiently transfected into WEHI-bcl-xL (Fig. 6). Due to the short half-life of the reporter plasmids after transfection into the WEHI-bcl-xL cells, the cells were cultured with anti-IgM for 24 h before being electroporated with the reporter plasmids. After transfection, the cells were then cultured for a further 24 h with or without anti-CD40 in the presence of anti-IgM before harvesting for reporter assays. Cross-linking of sIgM on WEHI-bcl-xL cells induced a decrease in wild-type E2F1 promoter activity when compared with untreated cycling cells (data not shown). Conversely, treatment of anti-IgM arrested cells with anti-CD40 resulted in an induction of E2F1 promoter activity. In contrast, the activity of a comparable promoter with mutated E2F sites was unchanged following cross-linking of sIgM or CD40. These observations further support the notion that E2F1 plays a central role in mediating negative and positive signals emanating from sIgM and CD40 and that the E2F sites located at the E2F1 promoter are important for this regulation.

Expression of the Adenoviral Protein E1A or E2F1 Overcomes the Anti-IgM Imposed G1/G0 Arrest—To determine whether p130 (or possibly other pocket proteins) is indeed an important downstream target of sIgM and CD40-derived signals in this system, we analyzed whether the oncoviral protein E1A, which disrupts pocket protein/E2F complex formation (60, 61, 22), could overcome the anti-IgM-induced G1 arrest. To this end, we transiently transfected WEHI bcl-xL with expression plasmids encoding either wild-type or mutant form of E1A, in conjunction with a plasmid expressing the CD8 T cell surface marker (47). After treatment with anti-IgM for 48 h, these cells were stained with a fluorescein isothiocyanate-coupled anti-CD8 monoclonal antibody, and their cell cycle profile was analyzed using propidium iodide staining (Fig. 7). As expected, treatment with anti-IgM caused an accumulation of cells in G1/G0 in
cells transfected with the empty expression vector. In contrast, expression of E1A induced a significant portion of cells to leave G0/G1 phase in the presence of anti-IgM, indicating that E1A can overcome the sIgM-induced cell cycle arrest and reinforcing the concept that the pocket proteins play an important functional role in mediating anti-IgM-induced cell cycle arrest in these cells. This is dependent on the capacity of E1A to sequester p130, because the mutant E1A defective in binding to pocket proteins was inactive. To examine the importance of E2F1 expression in overcoming anti-IgM-induced cell cycle arrest, we replaced E1A with E2F1 in the above experiment. The results showed that overexpression of E2F1 has the same effects as E1A, rendering WEHI-bcl-xL cells insensitive to cell cycle arrest imposed by sIgM stimulation (Fig. 7). Again, growth arrest by anti-IgM was unaffected by transfection with a vector expressing a mutant E2F1 that is unable to bind to DNA (43). This provides further evidence that E2F1 activity is a crucial component of signal transduction pathways emanating from the antigen receptor and CD40. Furthermore, the transient transfection results also imply that the induction of E2F1 activity following anti-CD40 treatment is a cause rather than a consequence of reentry into the cell cycle.

**DISCUSSION**

In this study, we used WEHI bcl-xL cells as a model to investigate how growth-inhibitory signals (via sIgM) or growth-promoting signals (via CD40) control the activity of E2F in immature B lymphoid cells. These cells arrest in G0/G1 follow-
ing a 48-h culture with anti-IgM and reenter the cycle rapidly following stimulation via CD40, even in the continuing presence of anti-IgM (Fig. 1). We show that ligation of the antigen receptor induced down-regulation of pRB and p107 and the accumulation of hypophosphorylated p130, which forms a transcriptionally repressive complex with E2F4/DP1 (Figs. 2 and 3). This would lead to inhibition of transcription of E2F-regulated genes and cell cycle arrest (42, 55). Stimulation of these arrested cells via CD40 induced hyperphosphorylation of p130 and consequent derepression of E2F-regulated genes, culminating in reentry into the cell cycle (Fig. 3). Our data further suggest that E2F1, a component of E2F activity, is one of the downstream targets of p130 (Figs. 4–6). To our knowledge this is the first description of the effects of physiologically important growth-regulatory stimuli on the regulation of E2F activity in lymphocytes. Moreover, this cell system also represents a unique model for studying positive and negative signaling, particularly in B lymphoid cells, whereby the cells could be reversibly induced to exit and reenter the cell cycle without undergoing apoptosis.

Our present findings are consistent with a recent study (62) showing that anti-IgM could induce hypophosphorylation of pRB and arrest cell cycle progression in human Burkitt lymphoma cell lines. Although we have also been able to observe the presence of pRB by Western blotting, no pRB/E2F DNA binding complex was detected in either cycling or anti-IgM arrested WEHI 231 cells, and the reason for this observation is unclear. However, it is notable that the progressive dephosphorylation of pRB in anti-IgM-treated cells is accompanied by down-regulation of the protein (Fig. 3). As a result, it may be that the only pRB that exists in these cells is in its hypophosphorylated form, which cannot bind to E2F. Another likely explanation for the absence of pRB/E2F complexes is that dephosphorylation of pRB coincides with down-regulation of E2F1, its binding partner to DNA. These two scenarios are not mutually exclusive and could jointly account for the lack of pRB-containing E2F complexes in growth-arrested WEHI-bcl-xL. Although expression of pRB has previously been demonstrated to be modulated in response to sIgM signaling in wild-type WEHI-231 (63, 64), it might not be involved in regulating E2F-dependent transcription activity in the system studied here.

It is apparent that there is an inverse correlation between the accumulation of the free E2F1/DP1 and the higher order p130/E2F complexes. Linking our observations with previous findings (42), we propose that in anti-IgM-treated cells, accumulation of underphosphorylated p130 forms a transcriptionally repressive complex with E2F4 and DP1, which causes inhibition of E2F1 gene transcription, down-regulation of E2F1 protein expression, and a subsequent decrease in transcriptionally active E2F1/DP1 complexes. Upon anti-CD40 treatment, p130 becomes phosphorylated and ceases to bind to E2F4 and DP1, resulting in derepression of the E2F1 promoter, with a consequent increase in E2F1 transcription and accumulation of E2F1 protein and free E2F1/DP1 complex. It is highly likely that E2F1 is not the only E2F-responsive gene regulated by p130 in response to ligation of sIgM and CD40. However, the study of its regulation serves to exemplify the molecular mechanisms whereby signals emanating from these receptors modulate E2F activity in B lymphoid cells. Notably, the expression levels of E2F1, p107, and pRB follow similar kinetics in anti-IgM- and CD40-treated cells (Figs. 3 and 4). Because both p107 and pRB are also E2F-responsive genes (65, 66), it is conceivable that the down-regulation of pRB and p107 after sIgM cross-linking could again be the result of repression of their respective promoters, through accumulation of the G0/G1-specific p130/E2F complexes. However, it is likely that other post-transcriptional mechanisms (67, 68) also contribute toward the down-regulation of E2F2-regulated gene products, including E2F1, pRB, and p107, following anti-IgM treatment. This speculation is supported by the observation (Fig. 4) that the E2F1 protein level declined rapidly after down-regulation of E2F1 gene transcription in response to anti-IgM treatment. It is also noteworthy that there is some variability between experiments in the level of p107 at 48 h anti-IgM, which seems to relate to the precise degree of cell cycle arrest in individual experiments. Although treatment of these cells with anti-IgM for a prolonged period would guarantee complete arrest, high levels of p130, and very low levels of p107, it also would compromise the ability of the cells to recover in response to anti-CD40.

In wild-type WEHI-231 cells, anti-IgM treatment in early G1 leads to growth arrest followed by apoptosis (9, 69). This has been shown to be accompanied by the accumulation of hypophosphorylated pRB (70) and inhibition of the up-regulation of cyclin A-dependent kinase activity (cdk2) required for G1/S transition (71, 72). In addition, these cells show elevated levels of the cdk inhibitor p27kip1, which is likely to play an important role in regulating the activity of cdk2 and consequent G1 arrest (71, 72). These observations are consistent with our present findings, because the pocket proteins, including pRB, p107, and p130, have been demonstrated to be direct phosphorylation targets of cyclin-dependent kinases. Although G1 arrest seems to be critical for the commitment of wild-type WEHI-231 to apoptosis, our study does not address the question of whether E2F activity plays a role in the apoptotic program. This question may warrant further investigation.

In conclusion, this study describes a direct link between two important B lymphocyte surface receptors and control of the cell cycle transcription machinery. These data should therefore provide a platform for future studies on the regulation of the cell cycle in normal B lymphocytes. We believe that the present system represents an in vitro model of antigen-induced anergy in B cells. Studies with double transgenic mice, the B cells of which express sIgs specific for hen egg lysozyme, as well as soluble hen egg lysozyme, have shown that anergy is induced in B cells as a result of chronic stimulation by poorly cross-linking antigen (2). The cells become abortively activated when they first encounter antigen in vivo, but then they disappear within a few days unless they encounter T cell help (73, 74). Anergic B cells have profound defects in signaling via their antigen receptors but can be induced to proliferate by stimulation via CD40 (75–78). A similar anergic state can be induced in vitro by treating immature B cells with suboptimal concentrations of anti-IgM (79). More recent studies have shown that these cells progress into the G1 phase of the cell cycle when cultured with anti-IgM and then undergo apoptosis (21). This is accompanied by up-regulation of the G1 cyclin D2 and one of its associated kinases, cdk4, but not the G1/S cyclin E or its partner cdk2 (21). No information is available on the levels of the various pocket proteins in these cells (although we have shown that mature, resting B cells express large amounts of p130 and negligible amounts of pRB or p107; data not shown) or on the induction of E2F activity following their activation. These questions will be the subjects of our future investigations.

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