Modulation of E2F Complexes during G₀ to S Phase Transition in Human Primary B-lymphocytes*

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Jeroen van der Sman‡, N. Shaun B. Thomas§, and Eric W.-F. Lam‡

From the §Ludwig Institute for Cancer Research and Section of Virology and Cell Biology, Imperial College School of Medicine at St Mary's, London W2 1PG and §Department of Haematology, University College London Medical School, London WC1E 6HX, United Kingdom

The pocket protein-E2F complexes are convergence points for cell cycle signaling. In the present report, we identified and monitored the pocket protein-E2F complexes in human primary B-lymphocytes after activation by phorbol 12-myristate 13-acetate. Consistent with previous data from human and mouse fibroblasts and T-lymphocytes, E2F4 and DP1 form the predominant E2F heterodimers both in G₀ and G₁ phases of the human B-lymphocyte cell cycle, whereas E2F1 and -3 are first detected in late G₁, and their expression levels increase towards S phase. Intriguingly, the major E2F complex that we detected in quiescent human B-lymphocytes is comprised of pRB, E2F4, and DP1. Though the levels of DP1 and -2 increase when cells progress from G₀ to S, the proportion of DP1 to DP2 remains relatively constant during the cell cycle. We also observed an increase in electrophoretic mobility of the predominant E2F components, DP1 and E2F4, as B-lymphocytes progressed from G₀ into early G₁. This increase in mobility was attributable to dephosphorylation, as a phosphatase treatment could convert the slower migrating forms into the corresponding faster mobility forms. We further demonstrated that this change in phosphorylation status correlates with a decrease in DNA binding activity. This modulation of DNA binding activity mediated through the dephosphorylation of DP1 and E2F4 could help to explain the lack of in vivo DNA footprinting in late G₁ and S phases of gene promoters negatively regulated through E2F sites and suggests a novel mechanism for controlling E2F transcriptional activity during the transition from quiescence to proliferation.

E2F is a transcription factor that controls cell proliferation through regulating the expression of essential genes required for cell cycle progression (1–4). The E2F transcription factor consists of one protein subunit encoded by the E2F family of genes and the other by the DP gene family, and to date, six distinct E2F (E2F1–6) and two DP (DP1–2) genes have been cloned from mammalian cells (4). The E2F and DP proteins cooperate to bind DNA and activate transcription of target genes in a synergistic manner (2). E2F activity is negatively regulated through interactions with the retinoblastoma protein (pRB) family of “pocket proteins,” consisting of pRB, p107, and p130 (5). Although all three pocket proteins repress E2F-dependent transcription (6–9), individual pocket proteins bind preferentially to particular subsets of E2F family members. Thus, pRB interacts exclusively with E2F1, -2, and -3 (10), p107 binds predominately to E2F4, and p130 binds specifically to both E2F4 and -5 (6–9, 11–14). Unlike E2F1–5, the newly identified member of the E2F protein family, E2F6, does not possess an equivalent pocket protein binding domain and therefore does not interact with the pRB family of proteins in the same manner as other E2Fs. The functional role of E2F6 has yet to be fully established, but the protein is believed to act as a repressor for E2F-dependent transcription (15–17).

It has been demonstrated that E2F binding sites can regulate cell cycle-dependent transcription through acting as transcriptional activators and/or repressors during different phases of the cell cycle. For instance, B-myb, E2F1, E2F2, cyclin E and cdc2 promoter activity appears to be cell cycle-regulated predominantly by repression through the E2F binding site during G₀ and early G₁ (18–24); in contrast, DHFR transcription is primarily activated through the E2F sites during late G₁ and S phase (25, 26).

The pocket proteins complex with E2F at distinct phases of the cell cycle. Thus, p130-E2F complexes are detected exclusively in G₁, and cells exiting G₀ (27, 28), pRB-E2F complexes exist predominantly in late G₁ and S phases, whereas complexes containing p107 are detected almost throughout the cell cycle. The pocket proteins are phosphoproteins, and their expression levels and phosphorylation states primarily determine their interaction with E2F during the cell cycle (29–33). pRB is hypophosphorylated in early G₁ and becomes progressively hyperphosphorylated toward late G₁ and S phases. The consequence of pRB hyperphosphorylation is the release of “free” E2F, which activates the transcription of E2F-regulated genes (34–36). However, exceptions to this general concept have also been reported, as some pRB-E2F complexes persist well into S and G₂ phases (37). In G₀ and early G₁, p107 is present at low levels in a hypophosphorylated form. As cells progress toward late G₁, the level of p107 increases, and the majority becomes hyperphosphorylated (29).

Hypophosphorylated forms of p130 are detected primarily in G₀ and early G₁ phases of the cell cycle. In mid-G₁, p130 becomes hyperphosphorylated, which persists for the rest of the cell cycle (32). Phosphorylation of p130 at mid-G₁ is believed to play an essential role in relieving E2F-mediated repression of G₁/S phase genes (7), including E2F1 and B-myb. However, p130 may not regulate E2F activity in continuously cycling cells as it is present at low levels and/or in a hypophosphorylated state (38, 39). Increasing evidence has shown that cyclins and their dependent kinases (cdks) associate with pocket proteins and are largely responsible for their phosphorylation in vivo (5, 40). Though previous mobility shift analyses have shown that the predominant E2F complex in S phase...
consists of p107, E2F4, DP1, and cyclin A (19–21, 37, 41), recent in vivo footprinting studies of the M-bmy, cyclin A, and cdc2 promoters have demonstrated that the corresponding E2F sites are only engaged by transcription factors in G0 and early G1 but are largely unoccupied in late G1 and S phases (42–44). The reason for this discrepancy is not yet understood, and further information is required on the molecular mechanisms regulating the occupancy of the endogenous E2F binding sites that can account for these in vivo protection patterns.

The E2F transcription factor, in conjunction with the retinoblastoma family of proteins, orchestrates the orderly progression of cell cycle regulatory proteins at specific points of the cell cycle and thereby controls cell cycle progression. Hence, the pocket protein-E2F complexes are convergence points of positive and negative proliferative signals. Although it has been shown that overexpression of E2F activity can drive cell lines from G0 (quiescence) into S (DNA synthesis) phase, the modulation of E2F activity that accompanies this cell cycle phase transition in primary cells under normal physiological conditions is not fully defined. In particular, little information is available for the regulation and roles of the DP family of proteins during this G0 to S transition, despite the wealth of knowledge relating to the E2F family proteins. Moreover, little is known about the roles of the E2F and pRB families of proteins during B-lymphocyte activation. The present report describes the modulation of E2F complexes during human B-lymphocyte activation through stimulation by PMA and explores the molecular mechanisms involved.

**Experimental Procedures**

**Isolation of Human Primary B-lymphocytes—**Platelet-depleted buffy coats obtained from the National Blood Transfusion Service were pooled, and the mononuclear lymphocytes were enriched using Ficol–Paque (Amersham Pharmacia Biotech) gradient centrifugation for 20 min at 800 \( \times \) g. The B-lymphocytes were isolated by positive selection using CD19-coupled Dynabeads (M-450 pan-B, Dynal). The B-lymphocytes captured by the magnetic beads were then released using Detachabeads (Dynal). As the control, small dense B-lymphocytes were also isolated by flow cytometric sorting from human tonsils, as described (45). The freshly purified B-lymphocytes were seeded at 10\(^6\) cells/ml in culture medium containing 15% fetal calf serum in RPMI and incubated for at least 36 h at 37 °C before stimulation by the addition of PMA at 30 µg/ml. Purity of B-lymphocytes was assessed by staining with fluorescein isothiocyanate (FITC)-conjugated anti-human B cell (CD20) antibodies (Dako) and assayed using flow cytometry.

**Flow cytometric analyses—**Cell cycle analysis was performed by propidium iodide and FITC staining as described previously (55). Cells were collected by centrifugation, washed with PBS before fixing in 20% PBS and 80% ethanol. The fixed cells were then washed with PBS and incubated with DNase free-RNase (0.5 mg/ml), propidium iodide (20 µg/ml), and FITC (0.05 µg/ml) for 30 min at 37 °C before analysis using a EPICS-Elite flow cytometer (Coulter, UK). For anti-CD20 staining, the cells were incubated with 1 µg/ml FITC-conjugated anti-human CD20 antibody (Dako A/S, Denmark) in PBS with 5% fetal calf serum for 30 min at 4 °C before fixing with ethanol/PBS.

**Electrophoretic Mobility Shift and Supershift Analyses of E2F DNA Binding Complexes—**Whole cell extracts from human B-lymphocytes were prepared as detailed previously (57). Protein yield was quantified by the Bradford method (Bio-Rad). E2F gel retardation assays were performed essentially as described (19), using a double-stranded oligonucleotide (5'-GATCTAGTTTTCGGCCTAAGTTTGA) containing the distal E2F binding site from the adenovirus type 5 E2a promoter (57).

**Western Blot Analysis and Antibodies—**Western blot extracts were prepared from B-lymphocytes by lysing with 4 times the packed cell volume of lysis buffer (20 mM HEPES, pH 7.9, 150 mM NaCl, 1 mM MgCl\(_2\), 5 mM EDTA, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 5 mM sodium orthovanadate) on ice for 20 min. Fifty µg of lysate was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and recognized by specific antibodies. The antibodies were detected using horseradish peroxidase-linked goat anti-mouse or anti-rabbit IgG (Dako) and visualized by the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech). Both the anti-CDP1 and DP2 monoclonal antibodies were raised against s6-His-tagged (Qiagen) peptides corresponding to unique amino-terminal regions of the corresponding human proteins. Antibodies against anti-E2F1(KH95), E2F2(-20), E2F3(-N20), E2F4(C-20), E2F5(MHS), pRB(-C-15), p107(C-18), and p130(C-20) were purchased from Santa Cruz Biotechnology. The monoclonal antibody against human c-MYC was prepared from supernatant of the hybridoma 9E10 (59).

**Phosphatase Treatment of Cell Extracts—**Dephosphorylation of cell extracts was performed by incubating whole cell extracts with 500 units of lambda protein phosphatase (New England Biolabs) at 30 °C for 1 h as detailed before (55). The reactions were stopped by boiling with SDS sample buffer, separated on SDS-polyacrylamide gels, and Western-blotted with the appropriate antibodies.

**Note that the anti-E2F4 polyclonal antibodies also recognized an unspecific band (indicated by asterisk in Fig. 5) in the whole cell extracts, which was not detected previously in the Western blot lysate. DNA Binding Assay—**DNA binding assays were performed as described previously (38). Briefly, the cells were collected and lysed on ice for 10 min in lysis buffer containing 0.5% Nonidet P-40 supplemented with protease and phosphatase inhibitors as detailed by Thomas (60).

**RESULTS**

**Cell Cycle Analysis of Human Primary B-lymphocytes after Stimulation with PMA—**Human B-lymphocytes were purified from platelet-depleted buffy coats by positive selection using CD19-conjugated immunomagnetic beads before preliminary enrichment over Ficoll density gradients. The purity of human B-lymphocytes isolated by this procedure was routinely more than 95%, as verified by staining using fluorochrome-conjugated CD20 antibodies (Fig. 1A). The purified human B-lymphocytes were then stimulated to enter the cell cycle using PMA, and their cell cycle status was monitored by flow cytometric analysis of both DNA and protein content, gauged by the levels of propidium iodide and FITC staining, respectively (Fig. 1B). Untreated B-lymphocytes had a 2N DNA and a low protein content, indicating that the majority of the unstimulated B-lymphocytes were in G0. Upon PMA stimulation, the first sign of increased cellular protein content (FITC staining) was observed at 12 h, indicating that cells were beginning to enter early G1 (47). Nevertheless, the majority of the cells traversed the G0/G1 boundary between 24 and 36 h. A small population of x-ray films. Supershift assays were performed by adding 1 µl of concentrated antibodies. Rabbit anti-pRB2/p130 and anti-cyclin A antisera were kindly provided by Dr. A. Giordano and Dr. J. Pines, respectively. Anti-pRB mouse monoclonal antibody 21C9 (58) was a generous gift from Dr. Sybille Mittnacht. Anti-CDP1, -E2F1, -E2F4 rabbit antisera and antibodies against peptides corresponding to the carboxy-terminal regions of the respective human proteins and have been described previously (49). The anti-CDP2 polyclonal antibodies were raised against peptides corresponding to unique regions at the carboxy-terminal end of the protein. Anti-E2F2 (L-20), anti-E2F3 (N-20), and anti-E2F5 (E19) were purchased from Santa Cruz Biotechnology.

**Western Blot Analysis of E2F Complexes in B-lymphocytes**—Western blot extracts were prepared from B-lymphocytes by lysing cells with 4 times the packed cell volume of lysis buffer (20 mM HEPES, pH 7.9, 150 mM NaCl, 1 mM MgCl\(_2\), 5 mM EDTA, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 5 mM sodium orthovanadate) on ice for 20 min. Fifty µg of lysate was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and recognized by specific antibodies. The antibodies were detected using horseradish peroxidase-linked goat anti-mouse or anti-rabbit IgG (Dako) and visualized by the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech). Both the anti-CDP1 and DP2 monoclonal antibodies were raised against s6-His-tagged (Qiagen) peptides corresponding to unique amino-terminal regions of the corresponding human proteins.

**Antibodies against anti-E2F1(KH95), E2F2(-20), E2F3(-N20), E2F4(C-20), E2F5(MHS), pRB(-C-15), p107(C-18), and p130(C-20) were purchased from Santa Cruz Biotechnology. The monoclonal antibody against human c-MYC was prepared from supernatant of the hybridoma 9E10 (59).**

**Electrophoretic Mobility Shift and Supershift Analyses of E2F DNA Binding Complexes—**Whole cell extracts from human B-lymphocytes were prepared as detailed previously (57). Protein yield was quantified by the Bradford method (Bio-Rad). E2F gel retardation assays were performed essentially as described (19), using a double-stranded oligonucleotide (5'-GATCTAGTTTTCGGCCTAAGTTTGA) containing the distal E2F binding site from the adenovirus type 5 E2a promoter (57). Twenty µg of whole cell extract was incubated with 1–2 ng of 32P-labeled oligonucleotide probe in the presence of 2 µg of sonicated salmon sperm DNA and 200 µg of a comparable unlabeled double-stranded oligonucleotide which contained E2F site (5'-GATCTAGTTTTCGGCCTAAGTTTGA) in a total volume of 20 µl at 30 °C for 15 min. The reactions were electrophoresed on 4% polyacrylamide gels in 0.33 × Tris-buffered EDTA at 4 °C. The gels were then dried and exposed to x-ray films. Supershift assays were performed by adding 1 µl of concentrated antibodies. Rabbit anti-pRB2/p130 and anti-cyclin A antisera were kindly provided by Dr. A. Giordano and Dr. J. Pines, respectively. Anti-pRB mouse monoclonal antibody 21C9 (58) was a generous gift from Dr. Sybille Mittnacht. Anti-CDP1, -E2F1, -E2F4 rabbit antisera and antibodies against peptides corresponding to the carboxy-terminal regions of the respective human proteins and have been described previously (49). The anti-CDP2 polyclonal antibodies were raised against peptides corresponding to unique regions at the carboxy-terminal end of the protein. Anti-E2F2 (L-20), anti-E2F3 (N-20), and anti-E2F5 (E19) were purchased from Santa Cruz Biotechnology.
PMA-stimulated cells began to enter S phase (DNA synthesis) at 60 h, which is indicated by an increase in their DNA content. It is also notable that with PMA stimulation alone, the majority of cells remained in G0/G1 after 72 h. The relatively long G1 made this cell system ideal for investigating the early phases of the cell cycle.

Changes in E2F DNA Binding Activity in Human Primary B-lymphocytes after Stimulation with PMA—To characterize the functional E2F complexes in human B-lymphocytes, mobility shift analysis was performed on extracts derived from PMA-stimulated cells. The mobility-shift analysis (Fig. 2A) identified at least five species of DNA binding complexes containing E2F (complexes A to E) by virtue of the difference in their mobility. Of these, two are free E2Fs (complexes D and E), and the other three are complexed E2Fs (complexes A, B, and C), shown previously also to contain the pRB-related pocket proteins. As we have shown previously (48), only one predominant species of E2F complex (complex B) was detected in unstimulated human B-lymphocytes. Upon PMA stimulation, the level of this complexed E2F (complex B) increased, reaching a peak at 30 h. However, as the cells progressed toward S phase, this E2F complex declined gradually and was eventually replaced by two other complexed E2Fs (complexes A and C) and two free E2F species (complexes D and E).

To identify the components of these E2F complexes, we performed antibody supershift experiments on extracts corresponding to B-lymphocytes at G0, G1, and S phases of the cell cycle. As shown in Fig. 2B (0 h), the anti-pRB antibody supershifted the majority of the E2F complex (complex B) found in the G0 phase of the cell cycle, whereas the anti-p130 antibody shifted less than 50% of this G0 complex, and the anti-p107 failed to shift this complex at all. The addition of anti-cyclin A antibody produced an unspecific band but failed to disrupt the G0 complex, indicating that cyclin A is not present in the G0 E2F complex in B-lymphocytes. It is notable that the anticyclin A used here does not supershift E2F complexes but disrupts E2F complexes containing cyclin A (49). The G0 complex could also be supershifted by the anti-E2F4 antibody but not antibodies against E2F1, -2, -3. Antibodies raised against DP1 could again shift the majority of the complex, and the remaining E2F complex could be shifted by different anti-DP2 antibodies (Fig. 2C). This indicates that though DP1 is the predominant DP protein present in these complexes, a low level of DP2 is also complexed with E2F4 and pRB or p130. Therefore, we propose that the predominant E2F complex (B) present in unstimulated, quiescent B-lymphocytes largely contains E2F4, DP1, and pRB or p130. This observation is in contrast to what has been reported to be present in quiescent cells of other
primary cell types. For example, in quiescent serum-starved mouse fibroblasts and in resting human primary T lymphocytes and CD34⁺ cells, the major E2F complex consists of E2F4 and DP1 complexed with p130 (9, 27, 41, 48, 50).

As the B-lymphocytes progressed into early G₁ (24 h), there was a general increase in the level of this pRB- or p130-containing E2F complex (complex B), although the components of this complex remained unchanged, as demonstrated by the supershift analysis (Fig. 2B). Notably, at this time, low levels of free E2F started to accumulate. The slower migrating free E2F (complex D) can be supershifted by anti-E2F4, whereas the higher mobility free E2F (complex E) can only be shifted by

![Fig. 2. Electrophoretic mobility shift analysis of E2F DNA binding complexes after PMA stimulation. A, whole cell extracts were prepared from human B-lymphocytes at times indicated after PMA stimulation. The extracts were then used for gel mobility shift experiments with a ³²P-labeled E2F oligonucleotide as probe. The positions of different E2F complexes are labeled A to E. B, antibody supershift analysis of components of E2F complexes at different cell cycle stages. Supershifts were performed using specific antibodies against pRB, p107, p130, cyclin A, DP1, and E2F1–5 as indicated on whole cell extracts prepared from cells at 0, 24, 42, 48, and 72 h after PMA stimulation. Band A in panel A consists mainly of p107-cyclin A-E2F4-DP1, band B contains primarily pRB and p130-E2F4-DP1, band C contains pRB-E2F1–3-DP1, band D contains E2F4-DP1, and band E contains E2F1–3 and 5-DP1. C, supershift analysis for the presence of DP2 in E2F complexes. The whole cell extract from unstimulated B-lymphocytes was supershifted with anti-DP2 polyclonal antibodies (DP2a, -b, and -c) in the presence of anti-DP1 antibody.
antibodies to E2F5 but not other E2Fs. In late G1/S phase (48 h, 72 h), the complexes containing pRB and p130 disappeared and were replaced by at least four different E2F complexes (Fig. 2A). Subsequent supershift analyses (Fig. 2B) showed that the slowest migrating E2F complex (complex A) contains predominantly p107, cyclin A, E2F4, and DP1, the faster complexed E2F (complex C) consists of pRB, DP1, and E2F1, -2, -3, or -4, the slower of the two free E2F (complex D) is composed of DP1 and E2F4, and finally, the fastest migrating complex (complex E) is made up mainly of DP1 complexed either with E2F1, -2, -3, or -5.

Although previous studies have shown that E2F1, -2, and -3 interact specifically with pRB and E2F4 and 5 with p107 and p130, we show here that E2F4 and DP heterodimers bind largely to pRB in B-lymphocytes in G0 and G1 phases of the cell cycle. Consistent with our findings is the previous observation that E2F4, DP1, and DP2 were present throughout the time course, and similar to E2F4, both DP1 and DP2 underwent electromobility changes when the cells exited G0. In unstimulated B-lymphocytes, the anti-DP1 antibody recognized a protein of approximately 52 kDa, which upon stimulation with PMA, increased its migration rate as cells entered G1 from G0 (12–48 h; Figs. 3 and 6) and persisted in this high mobility form throughout late G1 (48 h) and into S (72 h). We also observed a significant increase in total DP1 level as the cells traversed into late G1 (48 h). In G0, the anti-DP2 monoclonal antibody recognized 3 doublets of apparent molecular masses of approximately 55, 48, and 43 kDa, and the faster migrating forms of each proteins predominated as cells entered G1 (12–24 h; Fig. 3). This result is consistent with a previous report showing that DP2 exists in vivo as three related proteins of the above deduced molecular masses (51).

Expression of E2F Components after PMA Stimulation—To investigate the molecular mechanisms underlying these changes in E2F complexes, the expression of individual E2F components identified by the mobility shift supershift experiments was analyzed by Western blot analysis. Despite a wealth of knowledge on how different E2F proteins are regulated during the cell cycle, little information is available for their heterodimeric partners, DP1 and -2. Therefore, we have raised monoclonal and polyclonal antibodies specifically recognizing individual DP proteins. The Western blot results (Fig. 3) showed that E2F1, E2F3, E2F4, DP1, and DP2 were expressed in B-lymphocytes but at distinct stages of the cell cycle. However, although we could detect E2F2 and -5 in E2F complexes by supershift analysis, we were unable to document their expression by Western blotting. This is likely to be because of the fact that the mobility shift assays are more sensitive than Western blotting. E2F4 is present as cells traversed from G0 to S phase, but the relative abundance of the different forms of E2F4 changed during this period. In G0, E2F4 was visible by Western blot analysis as multiple bands with different electromobility (54–64 kDa). Upon stimulation with PMA, the slower migrating forms gradually disappeared, and the two fastest migrating species predominated for the rest of the time course. Like E2F4, DP1 and DP2 were present throughout the time course, and similar to E2F4, both DP1 and DP2 underwent electromobility changes when the cells exited G0. In unstimulated B-lymphocytes, the anti-DP1 antibody recognized a protein of approximately 52 kDa, which upon stimulation with PMA, increased its migration rate as cells entered G1 from G0 (12–48 h; Figs. 3 and 6) and persisted in this high mobility form throughout late G1 (48 h) and into S (72 h). We also observed a significant increase in total DP1 level as the cells traversed into late G1 (48 h). In G0, the anti-DP2 monoclonal antibody recognized 3 doublets of apparent molecular masses of approximately 55, 48, and 43 kDa, and the faster migrating forms of each proteins predominated as cells entered G1 (12–24 h; Fig. 3). This result is consistent with a previous report showing that DP2 exists in vivo as three related proteins of the above deduced molecular masses (51).

Fig. 3. Western blot analysis of E2F, DP, and c-MYC protein expression after PMA stimulation. Western blot extracts prepared from cells at times indicated after PMA treatment were separated on 10% SDS-polyacrylamide gels and immunoblotted with antibodies against E2F1-5, DP1, and DP2, as well as a monoclonal antibody against human c-MYC, 9E10 (59).
in their phosphorylation status. To determine whether these electrophoretic mobility changes are the result of dephosphorylation, whole cell lysates from B-lymphocytes at G₀ (0 h) and late G₁ (48 h) were incubated with λ phosphatase. Phosphatase treatment converted the slower migrating DP1 from unstimulated quiescent B-lymphocytes (Fig. 5, 1st lane) to a faster migrating form (2nd lane) with similar mobility to the DP1 protein detected at late G₁ and S (lane 3). Thus, DP1 is present in a hyperphosphorylated form in G₀ phase and becomes dephosphorylated as cells progress from G₀ into G₁ phase. Similar phosphatase treatment failed to produce a detectable electrophoretic mobility change in DP1 derived from cells in late G₁ phase (Fig. 5, 4th lane), suggesting that the DP1 protein present in late G₁ is likely to be an unphosphorylated form. Phosphatase treatment also turned E2F4 proteins from both G₀ and G₁ phases into a faster migrating form (Fig. 5). This dephosphorylated form of E2F4 had higher mobility than those species observed in either G₀ or G₁/S, indicating that E2F4 changes from hyperphosphorylated to hypophosphorylated forms as cells progress from G₀ into late G₁. Although these forms of E2F4 in late G₁ and S are hypophosphorylated, they are not unphosphorylated, as phosphatase treatment can further increase their mobility. Similarly, phosphatase treatment also resulted in the disappearance of the apparent higher molecular weight forms of each of the three DP2 doublets and in reciprocal increases in the levels of the corresponding lower molecular weight forms. These results are consistent with a previous report that all three DP2 polypeptides are phosphoproteins (51). Notably, the kinetics for the accumulation of the pRB- or p130-containing G₀/G₁ E2F complexes parallels the levels of the slower migrating hyperphosphorylated forms of DP1, DP2, and E2F4, implying that the phosphorylation states of these E2F components could have a role in regulating the DNA binding activity of the E2F complexes.

Fig. 4. Western blot analysis of pocket protein expression in B-lymphocytes after PMA treatment. A, extracts prepared from cells at different times after PMA stimulation were resolved on 5% SDS-polyacrylamide gels and analyzed by Western blotting with polyclonal antibodies against pRB, p107, and p130. The hyper- and hypophosphorylated forms of each pocket protein are represented by the top and bottom bands, respectively. B, extracts prepared from unstimulated small dense B-lymphocytes enriched by cell sorting (lane 1) and those from cells isolated using anti-CD19-conjugated magnetic beads (lane 2–4) were analyzed by SDS-polyacrylamide gel electrophoresis, followed by Western blotting. Lanes 2, 3, and 4 represent B-lymphocytes stimulated with PMA for 0, 36, and 48 h, respectively.

**DISCUSSION**

In the present study, we identified and monitored the pocket protein-E2F complexes in primary B-lymphocytes that had been induced to enter the cell cycle with PMA. We also further investigated the underlying mechanisms regulating the formation and disassembly of these E2F complexes. We have used human B-lymphocytes as a model for primary quiescent cells because they can be isolated to a high degree of purity and have an extended G₁ phase. Moreover, in contrast to T lymphocytes, which are well studied, little is known about the molecular mechanisms in B lymphocytes that regulate entry into the cell cycle.

It has previously been shown that B-lymphocytes can be activated by anti-CD19; therefore, it is possible that isolation of B-lymphocytes by positive selection with anti-CD19-coupled magnetic beads could potentially stimulate quiescent B-lymphocytes to enter the cell cycle. We have guarded against this

![Fig. 5. Phosphatase treatment of human B-lymphocyte extracts. Whole cell extracts derived from human B-lymphocytes at 0 and 48 h after PMA treatment were incubated with λ phosphatase at 30 °C for 1 h. After phosphatase treatment, extracts were Western-blotted with antibodies against DP1, DP2, and E2F4, as described earlier.](image-url)
possibility by incubating the freshly isolated cells for 36 h without stimulus to allow existing proliferative signals in these cells to decay. Several lines of evidence indicate that the B-lymphocytes isolated are in quiescent (G₀) phase. First, the immediate-early gene c-myc was not expressed in unstimulated cells but was induced rapidly after the cells were stimulated to enter the cell cycle by PMA. Second, in unstimulated cells, we were able to detect the presence of the p130-E2F2 complex, which has previously been shown to be unique to cells in G₀. Third, flow cytometric analysis showed that these cells have a low protein content and a 2N DNA content, indicative of cells resting in G₀. Finally, the B-lymphocytes prepared by negative selection also showed the same expression pattern and phosphorylation status for pRB and p130 as the cells isolated by positive selection using anti-CD19 beads. Thus, these cells represent a highly homogeneous population of quiescent cells at the start of the experiments.

Although previous reports have indicated that E2F4-containing E2F heterodimers preferentially complex with p107 and p130 (9, 11, 12, 14) and that p130 is the predominant pocket protein present in G₀ and early G₁ phases of the cell cycle (27, 28, 48, 55), our bandshift and supershift experiments showed that the major species of E2F complex in B-lymphocytes in G₀ and early G₁ phases of the cell cycle consists of pRB binding to E2F4 and DP1 or DP2 (Fig. 2). This observation is supported by the detection of high levels of hypophosphorylated (E2F binding) forms of pRB in G₀ and early G₁, as well as the observation that E2F complexes are increased in quiescent p130-deficient (-/-) T-lymphocytes (50). The reason for this is as yet unclear; however, it is likely to be because of the fact that both pRB and p107 are E2F-regulated genes, and their expression is negatively controlled by the presence of p130. Thus, the high levels of pRB observed in quiescent (G₀) human B-lymphocytes could be a consequence of the low levels of p130 expressed in these cells. A very similar scenario where high levels of pRB-containing E2F4 complexes coexist with low levels of p130-E2F4 complexes has also been reported in differentiated HL 60 cells after treatment with PMA (56).

Previous reports have shown that the expression levels and phosphorylation states of the pocket proteins are important for the formation of the transcriptional repressive pocket protein-E2F complexes in G₀ and early G₁. Our present data suggest that the accumulation of the pocket protein-E2F complexes also depends on the expression levels as well as the phosphorylation status of DP1 and E2F4. This suggestion is based on the results that the slower migrating hyperphosphorylated forms of DP1 and E2F4 bind DNA preferentially, and these hyperphosphorylated DP1 and E2F4 become progressively dephosphorylated as the cells progress from G₀ to late G₁, concomitant with a parallel decrease in levels of pRB-E2F or p130-E2F complexes. Moreover, during the initial G₀ to early G₁ transition, the kinetics for the accumulation in levels of pRB-E2F or p130-E2F complexes also coincides with the elevation of DP1 and E2F4 expression. In conjunction with previous published results, our findings lead us to postulate that in G₀ and early G₁, the hyperphosphorylated forms of DP1 and E2F4 tether the hypophosphorylated pocket proteins to E2F DNA-binding sites to repress E2F-dependent transcription. As the cells progress through G₁, DP1 and E2F4 become progressively hypophosphorylated, culminating in a loss of DNA binding activity and derepression of promoters negatively regulated through E2F sites. This concept is compatible with the in vivo footprinting results of the B-myb, cdc2, and cyclin A promoters showing that the E2F sites are occupied only in G₁/early G₂ but not late G₂/S phase (42–44). These in vivo protection patterns are at variance with the usual mobility shift results showing that the E2F sites are occupied predominantly by high levels of p107-E2F4-DP1-cyclin A complexes during S phase. The disparity is likely to be because of the fact that the low stringency conditions used for mobility shift assays can detect low affinity E2F complexes not normally found on these endogenous promoters. This also suggests that the in vitro DNA binding experiments do not necessarily truly reflect the binding in vivo. The significance of this regulatory mechanism, particularly in B-lymphocytes, is further highlighted by the observation that there is a lack of detectable free E2F complexes in G₀ and early G₁ phases, indicating that the pocket proteins are present in excess over the components of E2F in early phases of the cell cycle, and consequently, the DNA binding activity of E2F is rate-limiting for the formation of complexed E2F.

In summary, our present findings contribute toward defining the underlying mechanisms that modulate E2F activity and, therefore, cell cycle progression during the G₀ to S transition in general and in B-lymphocytes in particular. Through our study, we also uncover and provide evidence for a novel and potentially important mechanism by which E2F complexes are regulated during early stages of the cell cycle.
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REFERENCES

[References list not provided in the image]