Early Induction of Cyclin D2 Expression in Phorbol Ester-responsive B-1 Lymphocytes

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Summary

B-1 lymphocytes represent a distinct B cell subset with characteristic features that include self-renewing capacity and unusual mitogenic responses. B-1 cells differ from conventional B cells in terms of the consequences of phorbol ester treatment: B-1 cells rapidly enter S phase in response to phorbol ester alone, whereas B-2 cells require a calcium ionophore in addition to phorbol ester to trigger cell cycle progression. To address the mechanism underlying the varied proliferative responses of B-1 and B-2 cells, we evaluated the expression and activity of the G1 cell cycle regulator, cyclin D2, and its associated cyclin-dependent kinases (Cdks). Cyclin D2 expression was upregulated rapidly, within 2–4 h, in phorbol ester-stimulated B-1 cells, in a manner dependent on intact transcription/translation, but was not increased in phorbol ester-stimulated B-2 cells. Phorbol ester-stimulated cyclin D2 expression was accompanied by the formation of cyclin D2-Cdk4, and, to a lesser extent, cyclin D2-Cdk6, complexes; cyclin D2-containing complexes were found to be catalytically functional, in terms of their ability to phosphorylate exogenous Rb in vitro and to specifically phosphorylate endogenous Rb on serine780 in vivo. These results strongly suggest that the rapid induction of cyclin D2 by a normally nonmitogenic phorbol ester stimulus is responsible for B-1 cell progression through G1 phase. The ease and rapidity with which cyclin D2 responds in B-1 cells may contribute to the proliferative features of this subset.

Key words: B lymphocytes • B-1 cells • B-2 cells • cyclins • cyclin-dependent kinases

B-1 cells constitute a unique B lymphocyte subset, originally distinguished from conventional B (B-2) cells by low level expression of the pan-T cell surface glycoprotein, CD5, but now known to exhibit many additional characteristic features that are both phenotypic and functional in nature (for review, see references 1–3). B-1 cells appear early in development and contribute substantial proportions of nonimmune (resting) IgM and IgA that are repertoire restricted. Early adoptive transfer experiments suggested that B-1 cells represent a separate lymphocyte lineage whose precursors are not found in adult murine bone marrow (1–3). Instead, repopulation of B-1 cells occurred only in mice that had also received surface Ig (sIg)-positive B-1 cells, thereby defining the capacity of B-1 cells for “self-renewal.” Aberrations in this process may be associated with the occurrence of clonal expansions of B-1 cells (4, 5). More recent studies have raised the possibility that B-1 cells result from particular sIg signaling of a relatively mature B cell; this is supported by in vitro studies showing that B-2 cells acquire CD5 expression after sIg cross-linking, and in vivo studies demonstrating an overabundance of B-1 cells in mice transgenic for certain B cell receptors (6, 7). In keeping with this, B-1 cells bear some features of previously activated B cells, including low density, surface expression of CD44 and IL-5R, and nuclear, activated signal transducer and activator of transcription (STAT)1 and STAT3 (8–10). However, numerous other molecular and transcriptional markers for activation are lacking (11–13). Thus, regardless of origin, mature B-1 cells cannot be looked on simply as an activated version of B-2 cells, but rather appear to manifest a unique blend of characteristics, some of which are induced in B-2 cells after stimulation.

B-1 cells differ dramatically from B-2 cells in the signals required to produce cell cycle progression to S phase.
Cyclin D2 Expression in B-1 Cells

Materials and Methods

Animals. Male BALB/cByJ mice at 8–14 wk of age were obtained from The Jackson Laboratory. Mice were housed at least 1 wk before experimentation. Mice were cared for and handled at all times in accordance with National Institutes of Health and institutional guidelines.

B Cell Purification. B-1 and B-2 lymphocytes were prepared by negative selection from peritoneal washout cells and from spleen cell suspensions, as described previously (35). The resulting B cells were cultured at 37°C with 5% CO2 in RPMI 1640 medium (BioWhittaker) supplemented with 5% heat-inactivated fetal bovine serum (Sigma Chemical Co.), 10 mM Hepes (pH 7.2), 50 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. B-1 cells were 90–96% IgM+; CD5/Mac-1+ by flow cytometric analysis.

B cells were lysed by incubation for 30 min (4°C) in ice-cold NP-40 buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM EDTA, 0.5% NP-40, 1 mM PM SF, 25 μg/ml leupeptin/aprotinin, 1 mM Na3VO4, and 10 mM β-glycerophosphate) (22). Insoluble material was removed by centrifugation at 15,000 g for 15 min (4°C). Cell lysates were then incubated for 3 h with 1.5 μg nonimmune IgG or 1.5 μg anti-Cdk4 Ab, or 1.5 μg anti-Cdk6 Ab, followed by the addition of 50 μl of a 1:1 dilury of protein G–agarose. After 90 min, the immune complexes were collected, washed several times in NP-40 buffer, and separated by electrophoresis through a 10% polyacrylamide SDS gel. The resulting proteins were then transferred to Immobilon-P membrane (Millipore) and immunoblotted with an anti-cyclin D2 mAb (1:500 dilution in TBST) as described below.

Immunoblotting. For detection of cyclin D2, cyclin D3, and retinoblastoma, B lymphocytes were solubilized in 100 μl of salsubilization buffer (50 mM Hepes, pH 7.4, 15 mM EGTA, 137 mM NaCl, 15 mM MgCl2, 0.1% Triton X-100, 10 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM PM SF, and 1 μg/ml aprotinin/leupeptin) and NP-40 buffer supplemented with 20 mM NaF, respectively (36). Insoluble material was removed by centrifugation at 15,000 g (15 min), and 10–20 μg of total protein was separated through a 12% polyacrylamide SDS gel and transferred to Immobilon-P membrane. The Immobilon-P membrane was blocked in TBS (20 mM Tris, pH 7.6, 137 mM NaCl, and 0.1% Tween-20) containing 5% nonfat dry milk (4 h), washed several times, and then incubated 18 h with specific primary Abs. The membrane was washed extensively with TBS, incubated with anti-rabbit or mouse IgG-conjugated horseradish peroxidase Ab at 1:3,000 in TBS (90 min), and developed by enhanced chemiluminescence.

Immunoprecipitation. B cells were lysed by incubation for 30 min (4°C) in ice-cold NP-40 buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM EDTA, 0.5% NP-40, 1 mM PM SF, 25 μg/ml leupeptin/aprotinin, 1 mM Na3VO4, and 10 mM β-glycerophosphate) (22). Insoluble material was removed by centrifugation at 15,000 g for 15 min (4°C). Cell lysates were then incubated for 3 h with 1.5 μg nonimmune IgG or 1.5 μg anti-Cdk4 Ab, or 1.5 μg anti-Cdk6 Ab, followed by the addition of 50 μl of a 1:1 dilury of protein G–agarose. After 90 min, the immune complexes were collected, washed several times in NP-40 buffer, and separated by electrophoresis through a 10% polyacrylamide SDS gel. The resulting proteins were then transferred to Immobilon-P membrane (Millipore) and immunoblotted with an anti-cyclin D2 mAb (1:500 dilution in TBST) as described below.

In Vitro Rb Kinase Assay. The immune complexes were then washed six times with Rb buffer and three times in a buffer of 50 mM Hepes, 10 mM glycerophosphate, 1 mM Na3VO4, 1 mM PM SF, and 0.1% Tween-20 containing 5% nonfat dry milk (4 h), washed several times, and then incubated 18 h with specific primary Abs. The membrane was washed extensively with TBS, incubated with anti-rabbit or mouse IgG-conjugated horseradish peroxidase Ab at 1:3,000 in TBS (90 min), and developed by enhanced chemiluminescence.

Northern Blot Analysis. Total RNA was isolated from primary B cells (UltraSpec RNA reagent; Biotecx Laboratories, Inc.), size fractionated by denaturing agarose gel electrophoresis, and transferred to Genescreen Plus membranes (New England Life Science Products Inc.). Membranes were hybridized with radiolabeled cDNA probes specific for cyclin D2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), generated by PCR using previously reported primer sequences (37, 38), and developed by autoradiography.
cycin D2 expression (Fig. 1 A). The PMA-induced increase in cyclin D2 occurred quite early, reaching a peak within 2–4 h of treatment, much sooner than the onset of cyclin D2 expression in anti-Ig-stimulated B-2 cells, and significantly earlier than inducible cyclin D2 expression observed in other cells of hematopoietic origin (20, 23, 42). By 14 h, the level of cyclin D2 in PMA-stimulated B-1 cells had significantly declined although it was still readily detected as B-1 cells entered S phase.

PMA stimulation also produced an increase in cyclin D3 expression, but this occurred much later (at 14–24 h) and took place in both B-1 and B-2 cells (Fig. 1 B). Much of the delayed increase in cyclin D3 would appear to be too late to control B-1 G1-S transition, inasmuch as entry into S phase occurs at 18 h and peak S phase is found at 24–30 h of PMA stimulation (14, 15). Cyclin D1 expression was not stimulated by PMA in B-1 or B-2 cells (data not shown).

These findings indicate that cyclin D2 induction accurately reflects the divergent mitogenic responses of B-1 and B-2 cells, and strongly suggest that early cyclin D2 expression is a key feature of the B-1 cell S phase response to phorbol ester stimulation.

To determine whether the early induction of cyclin D2 depends on new protein synthesis and/or new gene expression, B-1 cells were treated with PMA in the presence or absence of cycloheximide and actinomycin D for 4 h. As shown in Fig. 2 A, both cycloheximide and actinomycin D completely blocked cyclin D2 expression induced by PMA. These results suggest that cyclin D2 expression in PMA-treated B-1 cells is regulated at the level of transcription. This conclusion is supported by Northern blot analysis showing marked induction of cyclin D2 mRNA expression after B-1 cell stimulation with PMA for 1 (data not shown) and for 2 h (Fig. 2 B).

To evaluate whether the early transcriptional induction of cyclin D2 is accompanied by the formation of cyclin D2-Cdk4 or cyclin D2-Cdk6 holoenzyme complexes, B-1 and B-2 cells were treated with PMA or anti-Ig for various

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**Results**

To investigate intrinsic differences between B-1 and B-2 proliferative responses to phorbol ester, we evaluated the expression of D-type cyclin regulators, which function to couple mitogenic pathways to cell cycle regulatory Cdks in a number of divergent cell types (18). Because we previously identified cyclin D2 as the major D-type cyclin expressed in anti-Ig and LPS mitogenically activated mature B-2 lymphocytes, we initially focused on this G1 cyclin (36).

B cells were treated with the phorbol ester PMA or anti-Ig for various periods of time, after which solubilized proteins were size fractionated by SDS-PAGE and immunoblotted with an mAb that specifically recognizes cyclin D2 (39). Stimulation of B-2 cells with anti-Ig produced substantial upregulation of cyclin D2 expression, which peaked at 24 h, as shown in Fig. 1 and as reported previously (36). In contrast, PMA treatment of B-2 cells, which fails to induce S phase entry, failed to produce any detectable increase in cyclin D2 (Fig. 1). The results with B-1 cells were completely inverted. Stimulation of B-1 cells with anti-Ig, which fails to induce S phase entry, failed to produce a substantial increase in cyclin D2 (data not shown). However, PMA treatment of B-1 cells produced marked induction of cyclin D2 expression.

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**Figure 1.** Cyclin D2 expression is induced early and uniquely in B-1 lymphocytes treated with PMA. (A) Primary B-1 and B-2 lymphocytes were cultured in the presence of medium alone (Med) or were stimulated with PMA (300 ng/ml) for 1, 2, 4, 14, and 24 h. As a control, parallel cultures of B-2 cells were treated with anti-Ig for 24 h. At the indicated times, cells were lysed in a 0.1% Triton X-100 buffer, and 10 μg protein was then resolved by 10% SDS-PAGE, transferred to Immobilon-P membrane, and immunoblotted with a cyclin D2-specific mAb. The position of cyclin D2 is indicated by the arrow on the right. (B) B-1 and B-2 cells were stimulated as indicated, after which cellular proteins were immunoblotted for expression of cyclin D3, as described above. The position of cyclin D3 is indicated by an arrow.

**Figure 2.** Induction of cyclin D2 expression in B-1 cells is regulated by transcription and translation. (A) Primary B-1 lymphocytes were cultured for 4 h in medium alone (Med) or in medium containing PMA at 300 ng/ml in the presence or absence of actinomycin D (ActD) at 500 ng/ml or cycloheximide (CHX) at 10 μg/ml. B cells were then collected and lysed in a 0.1% Triton X-100-containing solubilization buffer. Cellular proteins (10 μg) were resolved by 10% SDS-PAGE, transferred to Immobilon-P membrane, and immunoblotted with a cyclin D2-specific mAb as described in Methods. (B) Primary B-1 lymphocytes were cultured for 4 h in medium alone (Med) or in medium containing PMA at 300 ng/ml, and total RNA was extracted, resolved by denaturing agarose gel electrophoresis, and Northern blotted for expression of cyclin D2 and GAPDH as described in Methods. The positions of cyclin D2 and GAPDH mRNAs are indicated by arrows.
within 4 h and the extent of this phosphorylation increased．

Figure 3. Cyclin D2–Cdk4 complexes are formed in response to PMA stimulation in B-1 cells. B-1 and B-2 lymphocytes were cultured in medium alone (M) or in medium containing PMA at 300 ng/ml (P) for 4 h, after which non-denaturating NP-40 detergent lysates were prepared and immunoprecipitated with anti-cyclin D2 Ab. Immune complexes were recovered and assayed for in vitro kinase activity using a truncated p56h protein substrate as described in Materials and Methods. The position of p56h is indicated. (B) B-1 cells were cultured in medium alone (M ed), medium containing PMA at 300 ng/ml, or medium containing anti-Ig at 15 μg/ml (Ig), for the indicated times, and then solubilized in NP-40 lysis buffer. 20 μg of protein was separated by 10% SDS-PAGE, transferred to Immobilon-P membranes, and then immunoblotted with an anti-pRb Ser780 Ab to detect phosphorylation of endogenous Rb at the cyclin D–Cdk4 phosphoacceptor Ser780 site (reference 38).

Figure 4. Cyclin D2–containing complexes produced by PMA treatment of B-1 cells are active. Rb-phosphorylating kinases. (A) B-1 and B-2 cells were cultured in medium alone (M) or in medium containing PMA at 300 ng/ml (P) for 4 h, after which non-denaturating NP-40 detergent lysates were prepared and immunoprecipitated with anti-cyclin D2 Ab. Immune complexes were recovered and assayed for in vitro kinase activity using a truncated p56h protein substrate as described in Materials and Methods.

Discussion

We examined D-type cyclin expression in B-1 cells to elucidate the mechanism underlying the rapid onset of S phase produced by stimulation with phorbol ester alone. In keeping with previous results, cyclin D2 was found to be the major D-type cyclin induced by proliferative signals in B-1 and B-2 cells. PMA induced cyclin D2 early (at 2–4 h) in B-1 cells but not at measurable levels in B-2 cells, whereas in direct contrast, anti-Ig induced cyclin D2 late (at 24 h) in B-2 cells with little, if any, expression detectable in B-1 cells (data not shown). The phorbol ester–induced expression of cyclin D2 in B-1 cells is controlled at the level of transcription inasmuch as the PMA-stimulated increase in cyclin D2 protein (a) was blocked by actinomycin D, and (b) was accompanied by a rapid increase in cyclin D2 mRNA. Cyclin D3 was also induced after PMA stimulation, but this occurred much later and in both B-1 and B-2 cells. Thus, cyclin D2 expression appears to be an accurate, consistent, and early reflection of the competency of particular stimuli to induce cell cycle progression to S phase in discrete primary B cell populations. Moreover, the ease and rapidity with which a key cell cycle control protein is induced in B-1 cells may be causally related to the self-renewing characteristics of this B cell subset as well as its propensity for clonal and malignant transformation (1–6).
The unexpected induction of cyclin D2 by PMA alone, uniquely in B-1 cells, provides a molecular basis for the observation that PMA-stimulated B-1 cells progress to S phase entry, and this is supported by the demonstration that PMA-stimulated cyclin D2 associates with Cdk4 and results in the early appearance of Rb-phosphorylating activity. The induction of kinase-active cyclin D2-containing complexes in PMA-responsive B-1 cells provides an important demonstration that only mitogenic signals induce holoenzyme formation, in this case exemplified by B cell subsets that respond differently to the same stimuli. This greatly strengthens the role of cyclin D2-Cdk4 complex formation in B cell cycle progression, previously documented by treating B-2 cells with various stimuli that produce mitosis (36, 43–45).

It has been reported elsewhere that cyclin D2 is expressed early after murine splenic B cell (B-2 cell) stimulation (46). We do not find this to be so; instead, we find that the timing of cyclin D2 expression anticipates the timing of the S phase peak by ~24 h in both B-1 and B-2 cells (14, 15). The origin of the disparity in these sets of results remains uncertain, although it should be noted that in the study by Howard and colleagues, large, rather than small, B-2 cells were examined, which may reflect prior activation (46). However, the results we obtained are not simply a function of large size, inasmuch as there was little induction of cyclin D2 in B-1 cells stimulated by anti-Ig in our study (data not shown).

Our earlier observation that B-1 cells progress in cell cycle to S phase in response to phorbol ester treatment, whereas B-2 cells require treatment with a calcium ionophore in addition to phorbol ester, gave rise to the idea that B-1 cells endogenously express some signaling component or growth-promoting molecule that requires calcium ionophore for expression in B-2 cells. This notion is supported by our finding that B-2 cells stimulated with anti-Ig for 2 d become responsive to phorbol ester alone (47), further suggesting that a discrete alteration, inducible by sIg signaling in mature B-2 cells, is responsible for phorbol ester responsiveness. The present results suggest that this alteration, perhaps in the form of an sIg-triggered signaling component or growth-promoting molecule that is constitutively expressed in B-1 cells, relaxes (or fulfills one of) the requirements for cyclin D2 expression. Our recent finding that B-1 cells constitutively express nuclear, activated STAT3 that is triggered by PMA plus calcium ionophore (as well as by anti-Ig) in B-2 cells (10) suggests that one or more STAT proteins may play a role in regulating cyclin D2 expression.

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