The Normal Cell Cycle 

Abstract

B lymphocytes in the peripheral circulation are maintained in a non-proliferative state. Antigen recognition stimulates limited proliferation, whereas infection with Epstein-Barr virus (EBV) results in continual proliferation and the outgrowth of immortal cell lines. Because it is not clear at which point in cell cycle the peripheral B lymphocytes are arrested, we characterized the expression of several cell cycle-associated genes in quiescent and stimulated cells. We show that the expression of four cell genes, cdc-2, cyclin E, CD23, and cyclin D2, are up-regulated approximately 100-fold as a result of EBV-mediated immortalization. Because these genes play a positive role in cell proliferation, we suggest that this regulatory switch contributes to controlling entry into the cell cycle. Transient stimulation of quiescent B lymphocytes with either a cocktail of anti-CD40, anti-lgM, and IL-4, or EBV results in the rapid expression of the same four genes, suggesting that, after infection, EBV exploits the normal program of B-lymphocyte cell cycle activation.

Introduction

The B lymphocytes found in the peripheral circulation exist in a quiescent state and require stimulation with a specific antigen to enter into the cell division cycle and to proliferate. Although much progress has been made toward elucidating the signal transduction events arising from stimulation of the B-cell receptor (reviewed in Ref. 1), little is known about the molecular mechanisms that maintain the quiescent status of B lymphocytes, or about the changes necessary to facilitate cell cycle entry after stimulation. Recent progress in dissecting cell cycle controls has shown that there are many routes to exit from the cell cycle (2, 3), and it is not clear at which stage of the cell cycle B lymphocytes became growth arrested during their differentiation into peripheral cells.

Quiescent B lymphocytes can also be activated after infection with the DNA tumor virus EBV; infection causes cells to proliferate, and a proportion of the infected cells continue to divide indefinitely, resulting in the outgrowth of immortal LCLs (reviewed in Ref. 4). Mutagenesis experiments have shown that at least six viral genes are necessary for EBV-mediated immortalization (5), suggesting that a complex series of events are needed to override normal growth controls.

The simplest scenario to account for the quiescent phenotype of primary B lymphocytes would be the absence of essential positive regulators of cell cycle progression, such as cyclins or cdk's. We recently demonstrated that cyclin D2 mRNA is undetectable in quiescent primary B lymphocytes, but it is expressed at high levels after infection with EBV (6–8). Because the D-type cyclins, in conjunction with cdk4 and cdk6, act as positive regulators of progression through G1 (9–11), the increased expression of cyclin D2 in EBV-infected B lymphocytes might have a direct influence on their proliferation status.

In this report, we characterize the expression of several cell cycle control genes in quiescent B lymphocytes and investigate how these are altered in response to stimulation with physiological agents or infection with EBV. In addition to cyclin D2, we chose four cell genes involved in cell cycle control [cdc-2, cyclin E, c-myc, and B-myb (reviewed in Ref. 12)], as well as the B-lymphocyte activation antigen CD23. In its secreted form, CD23 acts as an autocrine growth factor for LCLs (13), and as a transmembrane protein it has the potential to act as a receptor; CD21 has been identified as one of its counter structures (14). Our data show that stimulation with physiological ligands or infection with EBV up-regulates the expression of the same genes, implying that the lack of expression of these genes is necessary to maintain the quiescent status of peripheral B lymphocytes.

Materials and Methods

Cell Culture. Primary B lymphocytes were isolated from adult peripheral blood by positive selection with pan-B-dynabeads essentially as described previously (7, 15). The lymphoblastoid cell line LCL#3 was generated by EBV-mediated immortalization of an equivalent population of cells (7). The cells were cultured in RPMI 1640 supplemented with 15% heat-inactivated FCS, penicillin, and streptomycin. Primary B cells were maintained at 1 X 10⁶/ml; LCL#3 cells were maintained at between 2 and 5 X 10⁶/ml. For infection experiments, EBV was purified (7, 15) from the B95-8 cell line. Its integrity was checked by the expression of viral genes or by the ability to stimulate DNA synthesis after infection (data not shown). B95-8 virus was added to primary B lymphocytes at saturating levels. For stimulation assays, BU.1 (anti-lgM; Ref. 16) was added to 25 μg/ml, G25-8 (anti-CD240; Ref. 17) was added to 1 μg/ml, and IL-4 (Sigma Chemical Co., Poole, United Kingdom) was added to 1000 units/ml; entry of cells into S phase was confirmed by measuring DNA synthesis (data not shown).

RT-PCR assays. Total cell RNA was routinely prepared from 1 X 10⁶ cells supplemented with 100 μg of yeast RNA (Boehringer Mannheim) to act as carrier. After treatment with DNase-1, random primed cDNA was prepared using a Stratascript RT-PCR kit (Stratagene) and was then used as a template for PCR (essentially as described; Ref. 6). The PCR reaction included 2 μM each dNTP supplemented with 1 μCi [32P]dCTP, and a standard thermocycle profile was used for all primer sets: 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min, for a total of 30 cycles. The products were fractionated on a 6% denaturing gel and visualized by autoradiography. The size of each gene product was confirmed by comparison with DNA size markers after fractionation on a 50-cm denaturing gel (data not shown). These genes included members of the cyclin family [cyclin D2 (8) and cyclin E (18)], the proto-oncogene cdk [cdc-2 (19)], growth-regulated transcription factors [c-myc (20, 21) and B-myb (22)], and the FcεRIα form of the B-lymphocyte activation antigen CD23 (23–25). The cell surface receptor CD44 (26) and the ribosomal phos...
The partial structure of the mRNA transcripts to be analyzed are shown on the left. mRNA; thick vertical lines, position of known splice junctions. The DNA sequences of the PCR primers used to amplify each gene are shown on the right; ---, position of the PCR product.

### COMMON PATHWAYS TO CELL CYCLE ACTIVATION

<table>
<thead>
<tr>
<th>Location of primers for RT-PCR</th>
<th>Size (bp)</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>cdc-2</td>
<td>231bp</td>
<td>FGGCTCCTGGAAATGTAGGCAGA3'</td>
</tr>
<tr>
<td>cyclin E</td>
<td>234bp</td>
<td>FGCTCCTTCTCCTTTAGGGCTT3'</td>
</tr>
<tr>
<td>CD23</td>
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<td>c-myc</td>
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<td>B-myb</td>
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</tr>
<tr>
<td>CD44</td>
<td>480bp</td>
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</tr>
<tr>
<td>36B4</td>
<td>180bp</td>
<td>FCGAGAAGCTGTAGTGGACGACGAG3'</td>
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</tbody>
</table>

### Results and Discussion

The increase in the level of cyclin D2 mRNA after EBV infection of primary B lymphocytes was shown to be at least 100-fold, as determined by a quantitative RNase protection assays (7). We therefore explored the possibility of using semiquantitative RT-PCR assays to detect equivalent changes in the expression of other cell cycle genes. A series of specific primers as chosen to amplify segments of each gene from randomly primed cDNA. In cases where the genomic organization was known, at least one splice junction was included in the amplified product (Fig. 1).

RNA was prepared from a population of quiescent primary B lymphocytes isolated from peripheral blood by positive selection (7, 15) and from a continually proliferating cell line established from an equivalent population of cells by EBV-mediated immortalization (LCL#3; Ref. 7). Using RT-PCR, we confirmed that the two control genes (the cell surface protein CD44 and the ribosomal phosphoprotein 36B4) were expressed at similar levels in primary B lymphocytes and LCL#3, with 36B4 up-regulated 10-fold after immortalization (Fig. 2). However, even with this sensitive assay, most of the other genes were not expressed at detectable levels in the primary B lymphocytes. This suggests that the peripheral B lymphocytes have entered into G0 and thus are in a similar state of quiescence to peripheral T cells and serum-starved fibroblasts. The only exception was the low level expression of c-myc mRNA, in line with a previous report (28). As anticipated, c-myc expression was up-regulated at least 10-fold in the LCL. The expression of B-myb mRNA also appeared to be increased by at least 10-fold. However, the most striking differences were in the expression of cdc-2, cyclin E, CD23, and cyclin D2; comparison of a cDNA dilution series suggested that the expression of these genes was increased at least 100-fold after EBV-mediated immortalization (Fig. 2).

The magnitude of these changes in gene expression, together with the ability to readily detect the differences using RT-PCR, may enable us to investigate the effects on cell cycle progression of introducing individual EBV genes into quiescent primary B lymphocytes (7). In addition, it will be interesting to investigate the direct or indirect mechanisms by which the cell genes described here are regulated by

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**Fig. 1.** Strategy for analysis of gene expression. The partial structure of the mRNA transcripts to be analyzed are shown on the left. mRNA; thick vertical lines, position of known splice junctions. The DNA sequences of the PCR primers used to amplify each gene are shown on the right; ---, position of the PCR product.

**Fig. 2.** Induction of gene expression after EBV-mediated immortalization of primary B lymphocytes. Total cell RNA was isolated from primary peripheral B lymphocytes and a lymphoblastoid cell line (LCL#3). RT-PCR assays for the indicated genes were carried out on a cDNA dilution series; the products were fractionated on a 6% denaturing gel and visualized by autoradiography.
EBV. It has been shown that the expression of CD23 and cyclin D2 are regulated by EBV genes (6–8, 29, 30) and, furthermore, that the increase in CD23 expression involves direct transcriptional regulation via an EBNA-2 response element within the promoter (31).

During the early stages of EBV-mediated immortalization, infected cells leave G0 and take 3–4 days to progress around the initial cell division cycle (32–34). To determine whether cdc-2, cyclin E, CD23, and cyclin D2 are early targets for EBV, we investigated their expression 4 days after infection with EBV. 36B4 mRNA was detected in both infected and noninfected cells and can be considered as a control for input RNA (Table 1). Increased expression of cdc-2, cyclin E, CD23, and cyclin D2 all occur within this period, thus, the up-regulation of these genes can be considered as events that occur during the initial cell cycle (Table 1 and Refs. 7 and 28).

The normal program of B-lymphocyte cell cycle activation can be mimicked in vitro by stimulating quiescent primary B lymphocytes with a variety of agents that cause limited cell proliferation (reviewed in Ref. 13). Because several features of this program are common to the early stages of EBV-mediated immortalization (32–34), we explored the possibility that cdc-2, cyclin E, CD23, c-myc, and cyclin D2 may also be up-regulated during this process. We therefore investigated the expression of the same genes 4 days after stimulation of quiescent B lymphocytes with a cocktail of physiological stimuli (anti-CD40, anti-IGM, and IL-4, Ref. 16) that are capable of driving quiescent B lymphocytes through at least one cell cycle. 36B4 mRNA was detected in both stimulated and unstimulated cells, but cdc-2, cyclin E, CD23, c-myc, and cyclin D2 mRNA were only readily detected in response to stimulation (Table 2).

In this report, we investigated changes in the expression of a set of positive regulators of cell cycle after in vitro activation with either physiological stimuli or EBV. From this analysis, we conclude that the positive regulators of cell cycle after in vitro activation with either physiological stimuli or EBV (Tables 1 and 2). Taken together with the observation that this quiescent B lymphocytes through at least one cell cycle. 36B4 mRNA was detected 4 days after infection with EBV. 36B4 mRNA was detected in response to stimulation (Table 2).

Table 1 Cell genes induced in the early stages of EBV infection

<table>
<thead>
<tr>
<th>Gene</th>
<th>Infection with B95-8 EBV&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>(-)</td>
</tr>
<tr>
<td>cdc-2</td>
<td>-</td>
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<td>cyclin E</td>
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<td>cyclin D2</td>
<td>-</td>
</tr>
<tr>
<td>36B4</td>
<td>-</td>
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</table>

<sup>a</sup> Primary B lymphocytes were incubated with B95-8 EBV where indicated and harvested after 4 days.

The expression of the indicated cell genes was determined by RT-PCR assay as described in Fig. 2.

Table 2 Cell genes induced in the early stages of B-lymphocyte activation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Stimulation with physiological agents&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
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<sup>a</sup> Primary B lymphocytes were incubated with BU.1, G25-8, and IL-4 where indicated and harvested after 4 days.

The expression of the indicated cell genes was determined by RT-PCR assay as described in Fig. 2.

For critical review of the manuscript.

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References

COMMON PATHS TO CELL CYCLE ACTIVATION


The Normal Cell Cycle Activation Program Is Exploited during the Infection of Quiescent B Lymphocytes by Epstein-Barr Virus


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