Suppression of c-Myc-Induced Apoptosis by the Epstein-Barr Virus Gene Product BHRF1

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Constitutive expression of the c-myc proto-oncogene in growth factor-deprived fibroblasts promotes proliferation and induces apoptosis. In these cells, apoptosis can be inhibited by survival factors such as insulin-like growth factor I or the bcl-2 proto-oncogene product. Deregulated c-Myc expression is a common feature in Epstein-Barr virus-positive Burkitt's lymphoma in which the c-myc gene is reciprocally translocated and placed under the control of one of the immunoglobulin loci. BHRF1 is an Epstein-Barr virus protein expressed early in the lytic cycle. BHRF1 is a member of the Bcl-2 family and has been shown to suppress apoptosis and to increase cell survival in different settings. In the present study, we report that BHRF1 inhibits c-Myc-induced apoptosis which occurs in the absence of survival factors. It does not, however, affect the capacity of c-Myc to promote cell growth. These findings demonstrate that BHRF1 has not only structural but also functional similarities to Bcl-2.

The Epstein-Barr virus (EBV) is a B-lymphotropic human herpesvirus associated with human lymphoproliferative diseases such as Burkitt’s lymphoma (BL) and Hodgkin’s disease. A characteristic feature of EBV is its ability to infect resting B lymphocytes in vitro, converting them into permanently growing, immortalized lymphoblastoid cell lines (LCLs) (reviewed in reference 29). BHRF1 is an EBV gene expressed early in the EBV lytic cycle. The BHRF1 protein shares 38% primary amino acid sequence homology with the bcl-2 proto-oncogene product over its carboxy-terminal region (11, 35, 36). Members of the Bcl-2-related family share three highly conserved domains: BH1, BH2 (38), and BH3 (6, 9). Like Bcl-2, BHRF1 possesses a C-terminal hydrophobic region which localizes it to intracellular membranes (19), primarily to the mitochondrial periphery in transfected cells (21). Ectopic expression of BHRF1 extends the survival of a murine interleukin-3 (IL-3)-dependent hemopoietic cell line (32D) upon removal of IL-3 (31). BHRF1 is also able to protect serum-deprived or ionomycin-treated BL cell lines (19) from cell death and to inhibit cisplatin-, etoposide-, and mitomycin-induced apoptosis in CHO cells (32). In these ways, BHRF1 is functionally similar to Bcl-2. It is worth noting that both the latency membrane protein 1 (LMP-1) and EBNA-2 of EBV have been shown to upregulate Bcl-2 expression and inhibit apoptosis in B cells (16, 20). This suggests that EBV may have evolved mechanisms to overcome the killing of host cells by expressing a set of antiapoptotic proteins, thereby allowing viral propagation. The c-myc gene is the cellular homolog of the viral oncogene c-myc found in a number of avian and feline retroviruses that induce leukemia, carcinomas, and sarcomas. Its expression has been associated with cell proliferation and neoplasia (12, 30). Deregulated c-Myc expression is frequently associated with BL, a B-cell neoplasia resulting from the translocation of the c-myc gene from chromosome 8 to chromosome 2, 14, or 22 (24). The most frequent translocation, t(8; 14), juxtaposes the c-myc gene locus on chromosome 8 to the immunoglobulin heavy chain gene locus on chromosome 14. This translocation places c-myc under the immediate control of the immunoglobulin promoter.

Deregulated expression of c-Myc accelerates apoptosis in serum-starved and drug-arrested fibroblasts (14) and in IL-3-dependent myeloid cells upon removal of IL-3 (3). c-Myc-induced apoptosis can be inhibited by Bcl-2 protein (5, 15, 34). The proto-oncogene bcl-2 is activated in the majority of non-Hodgkin’s lymphomas as a consequence of t(14; 18) translocation which juxtaposes the bcl-2 gene adjacent to the immunoglobulin G locus during immunoglobulin gene rearrangement in pre-B cells (4, 10, 33). The oncogenic activity of Bcl-2 does not result from an increase in c-Myc-induced proliferation but rather from an inhibition of the apoptotic function of c-Myc (15). This functional interaction between c-Myc and Bcl-2 exemplifies a novel form of oncogene cooperation. In order to test whether BHRF1 interferes with c-Myc cellular activities in a manner similar to Bcl-2, we investigated its effect on the proliferative and apoptotic functions of c-Myc.

BHRF1 inhibits c-Myc-induced apoptosis. Constitutive expression of c-myc or ectopic activation of the c-Myc-estrogen receptor fusion protein (13) induces apoptosis in serum-deprived Rat-1 fibroblasts (14, 15, 17). In the absence of serum, Rat-1/c-Myc-ER cells downregulate endogenous c-Myc, arrest in G0/G1, and remain viable for many days. Addition of exogenous β-estradiol activates c-Myc-ER and induces the rapid onset of apoptosis in Rat-1 fibroblasts (14). To test the effect of BHRF1 on the apoptotic and growth-promoting activities of c-Myc, we infected Rat-1/c-Myc-ER cells with the retrovirus vector pBabePuro (28) carrying bhrf1 cDNA. Clones of Rat-1/c-Myc-ER cells stably expressing bhrf1 mRNA were isolated after selection with 5 μg of puromycin/ml. Northern blot analysis using a 32P-labeled bhrf1 cDNA as probe (8) shows the expression of bhrf1 mRNA in two representative Rat-1/c-Myc-ER/BHRF1 clones, no. 2 and 4 (Fig. 1). Using these two clones, we first analyzed the effect of BHRF1 on c-Myc-induced apoptosis in G0/G1, and remain viable for many days. Addition of exogenous β-estradiol activates c-Myc-ER and induces the rapid onset of apoptosis in Rat-1 fibroblasts (14). To test the effect of BHRF1 on the apoptotic and growth-promoting activities of c-Myc, we infected Rat-1/c-Myc-ER cells with the retrovirus vector pBabePuro (28) carrying bhrf1 cDNA. Clones of Rat-1/c-Myc-ER cells stably expressing bhrf1 mRNA were isolated after selection with 5 μg of puromycin/ml. Northern blot analysis using a 32P-labeled bhrf1 cDNA as probe (8) shows the expression of bhrf1 mRNA in two representative Rat-1/c-Myc-ER/BHRF1 clones, no. 2 and 4 (Fig. 1). Using these two clones, we first analyzed the effect of BHRF1 on c-Myc-induced apoptosis. Rat-1/c-Myc-ER/BHRF1 clones and mock-infected Rat-1/c-Myc-ER cells were rendered quiescent by culturing them for 2 days in serum-free medium. c-Myc was then activated by the addition of 2 μM β-estradiol, and apoptosis was analyzed by time-lapse videomicroscopy as previously described (14, 15, 17). Under these conditions, BHRF1 is a potent inhibitor of c-Myc-induced apoptosis (Fig. 2). Eight clones...
constitutively expressing BHRF1 were tested, and all showed a substantial inhibition of c-Myc-induced cell death (not shown). Thus, BHRF1 protein is functionally similar to Bcl-2 (15).

BHRF1 does not affect the growth-promoting activity of c-Myc. We have previously shown that Bcl-2 inhibits the apoptotic function of c-Myc without affecting its mitogenic activity (15). We therefore sought to test whether BHRF1 affects c-Myc-induced proliferation in Rat-1 fibroblasts. Rat-1/c-Myc-ER/BHRF1 lines and control Rat-1/c-Myc-ER cells were rendered quiescent by serum starvation; then c-Myc was activated by the addition of 2 μM β-estradiol. Individual cell first divisions were scored by time-lapse videomicroscopy as previously described (14), and the results are presented as cumulative cell divisions versus time. Figure 3 shows the mitotic rate of Rat-1/c-Myc-ER cells in either the presence or absence of BHRF1. Mitotic rates of control Rat-1/c-Myc-ER cells and those expressing BHRF1 are similar, suggesting that BHRF1 does not significantly affect the growth-promoting activity of c-Myc.

Inhibition of c-Myc-induced death by BHRF1 protein in thymidine-arrested Rat-1 fibroblasts. The c-Myc protein is a potent inducer of apoptosis in Rat-1 fibroblasts which have been blocked in S phase with an excess of thymidine (14). This type of cell death can be inhibited by Bcl-2 (15). We therefore tested whether BHRF1 can inhibit c-Myc-induced apoptosis in thymidine-blocked fibroblasts. Rat-1/c-Myc-ER cells constitutively expressing BHRF1 as well as control cells were incubated with 2 mM thymidine for 24 h. Under these conditions, more than 96% of the cells arrested in S phase (results not shown), demonstrating that, as for Bcl-2 (15), BHRF1 does not relieve the cell cycle block imposed by thymidine. Activation of c-Myc in thymidine-arrested Rat-1 fibroblasts by the addition of 2 μM β-estradiol led to rapid cell death in control cells (Fig. 4). However, constitutive expression of BHRF1 in Rat-1/c-Myc-ER/BHRF1 cells substantially delayed and reduced the apoptotic function of c-Myc.

In this study, we analyzed the effects of the EBV BHRF1 protein on the proliferative and the apoptotic functions of c-Myc. EBV can infect resting B cells in vitro, leading to the generation of LCLs. In vivo, EBV is known to cause infectious mononucleosis. BHRF1 protein exhibits a peak of expression early in the EBV lytic cycle and is also transiently expressed in latently infected lymphoid cells (27). To date, in vitro studies of the role of BHRF1 in LCL development and virus replication have been inconclusive (26, 27). As previously suggested (19), BHRF1 may play a role in the survival of EBV-infected cells and the development of EBV-related tumors in vivo. In addition, deregulated c-Myc expression is a common event in BL, resulting from translocations involving c-myc and one of the
three immunoglobulin loci (24). The result is an excessive expression of c-Myc protein in B cells, preventing them from leaving the cycling state. Moreover, transgenic mice containing an ac-
myc gene linked to the Eμ immunoglobulin enhancer develop B-cell tumors (1, 18). c-Myc has also been shown to be a potent inducer of apoptosis in fibroblasts deprived of serum or treated with various drugs (14). Our results demonstrate that BHRF1 is a powerful inhibitor of c-Myc-induced apoptosis in serum-starved or drug-treated fibroblasts but has no effect on the mitogenic activity of c-Myc. It is not clear whether the cooperative interaction between c-Myc and BHRF1 could result in oncogenic transformation of EBV-infected cells.

It remains to be established precisely how c-Myc and
BHFR1 regulate the apoptotic machinery. Recent evidence suggests that c-Myc-induced apoptosis requires the interaction of Fas and Fas ligand on the cell surface (23). Two mechanisms have been proposed to explain the inhibition of cell death by Bcl-2 and its antiapoptotic homologs. The first model hypothesizes that antiapoptotic proteins such as Bcl-2 and Bcl-X\(_\text{L}\) are able to prevent the release of cytochrome \(c\) from the mitochondria into the cytosol (25, 37). A second model suggests that Bcl-2 and Bcl-X\(_\text{L}\) regulate the activation of caspases (2, 7). In this context, a recent study has demonstrated that Bcl-X\(_\text{L}\) binds to Apaf-1, a mammalian protein that shares homology with Caenorhabditis elegans CED-4, and inhibits the activation of caspase 9 (22). It is tempting to speculate that BHFR1 may act similarly by affecting caspase activation and/or cytochrome \(c\) release.

REFERENCES