Interferon Regulatory Factor 4 Is Involved in Epstein-Barr Virus-Mediated Transformation of Human B Lymphocytes

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Epstein-Barr virus (EBV) infection has been associated with many human malignancies. In vitro, EBV transforms primary B lymphocytes into continuously growing lymphoblastoid cell lines. EBV latent membrane protein 1 (LMP-1) is required for EBV transformation processes. Interferon regulatory factor 4 (IRF-4) is a transcription factor and has oncogenic potential. We find that high levels of IRF-4 are associated with EBV transformation of human primary B cells in vitro and with EBV type III latency in which LMP-1 is expressed. We show that EBV LMP-1 stimulates IRF-4 expression in B lymphocytes. The stimulation of IRF-4 by LMP-1 requires signaling from LMP-1 and involves cellular NF-kB. The growth of EBV-transformed cells is inhibited when IRF-4 is specifically down-regulated. We further demonstrate that IRF-4 knockdown cells have lower proliferation but higher apoptotic rates than control cells. Finally, IRF-4 is expressed in significant numbers of specimens of primary central nervous system (CNS) lymphomas (12/27 [44.4%]), an EBV-associated malignancy. The association between the expression levels of LMP-1 and IRF-4 is statistically significant (P = 0.011) in these CNS lymphomas. Our data suggest that IRF-4 may be a critical factor in EBV transformation and a useful target in the therapy of EBV-mediated neoplasia.

Epstein-Barr virus (EBV) infection has been associated with the development of several human malignancies, including nasopharyngeal carcinoma, Burkitt’s lymphoma (BL), Hodgkin’s lymphoma, T-cell lymphoma, and gastric carcinoma (32, 48). In immunocompromised individuals such as organ transplant recipients and AIDS patients, EBV almost certainly triggers two fatal cancers without the necessity for cofactors: AIDS-associated central nervous system (CNS) lymphoma and post-transplantation lymphoproliferative disorder (46).

EBV establishes several types of latencies in host cells. In type I latency, EBV establishes nuclear antigen 1 (EBNA-1) and small EBV-encoded, nonpolyadenylated nuclear RNAs (EBER-1 and -2) are expressed in host cells. In contrast, six nuclear proteins (EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, and EBNA-LP) are expressed in type II latency (32, 48).

EBV transforms adult primary B cells into continually growing lymphoblastoid cell lines (LCLs) and concomitantly establishes type III latency in vitro. LMP-1 is required for the transformation process: the deletion of LMP-1 prevents the transformation of primary B cells (27, 31), and the inhibition of LMP-1 expression in EBV-transformed cells reverts the transformed phenotypes (33). LMP-1 is an integral membrane protein with transmembrane domains and a C-terminal domain located in the cytoplasm (32, 35). LMP-1 acts as a constitutively active, receptor-like molecule that activates signaling pathways without the binding of a ligand (19). In addition, LMP-1 appears to be a central effector of altered cell growth, survival, adhesive, invasive, and antiviral potential (15, 41, 62, 63, 65, 69, 71).

Interferon (IFN) regulatory factors (IRFs) are a small family of transcription factors with multiple functions. IRFs are apparently associated with viral transformation. IRF-7 is associated with EBV-transformed CNS lymphomas and has oncogenic properties (78). Oncogenic human herpesvirus 8 (HHV-8), also called Kaposi’s sarcoma-associated herpesvirus, encodes four IRF-like molecules (viral IRFs [vIRFs]). Rhesus rhadinovirus, another oncogenic herpesvirus, has eight vIRFs in the genome (3, 47). At least human herpesvirus 8 vIRF-1 causes oncogenic transformation (17).

IRF-4, also known as LSIRF, ICSAT, Pip, and Mum1, was cloned independently as a homologous member of the IRF gene family (67) and as an interacting partner of PU.1 (14). IRF-4 is expressed at all stages of B-cell development, in mature T cells, and in macrophages. The analysis of mice lacking IRF-4 (IRF-4−/−) revealed that IRF-4 is essential for the function and homeostasis of both mature B and T lymphocytes (42). IRF-4 is a critical factor for pre-B-to-B transition and the development of certain dendritic cells (37, 58). In addition, IRF-4 is closely associated with the human T-cell leukemia virus transformation process (67), has oncogenic potential in vitro, and may prevent apoptosis (26, 36). IRF-4 is also implicated in the pathogenesis of multiple myeloma: some myeloma cells express high levels of IRF-4 resulting from the chromosomal translocation of the IRF-4 gene (26).

In this report, we have examined the role of IRF-4 in the EBV transformation process. The expression of IRF-4 is associated with the EBV transformation of primary B lymphocytes in vitro and with primary CNS lymphomas in vivo. The reduced expression of IRF-4 in EBV-transformed cells decreases the
cell proliferation rate and enhances apoptosis. These data suggest that IRF-4 may be a critical factor in the EBV-mediated transformation process.

MATERIALS AND METHODS

Plasmids and antibodies. Expression plasmids of LMP-1 and its signaling defective mutant, LMP-DM, were described previously (77). An expression plasmid of EBNA-2 (pAg155) was a gift from Paul Ling. The IκB expression plasmid and NF-κB reporter construct were gifts from Albert Baldwin. shLuc plasmid and NF-κB expression mid of EBNA-2 (pAG155) was a gift from Paul Ling. The IκB defective mutant, LMP-DM, were described previously (77). An expression plasmid of EBNA-2 was obtained from PBL Biomedical Laboratories (catalog number 41100).

Cell culture, transient transfection, and isolation of transfected cells. DG75 is an EBV-negative BL cell line (6). BL41 is an EBV-negative BL line; BL41-EV was generated by in vitro infection of BL41 with EBV strain B95-8 (10). Sav I and Sav III cells have the same cellular background but harbor different strains of EBV with different latencies (44). P3HR1 cells are derived from Jijoye cell line, and both cell lines are EBV positive (2). The Jijoye cell line has all the latency genes in its viral genome, whereas the P3HR1 cell line lacks the EBNA-2 gene and a portion of EBNA-LP (2). As a result of the deletion, P3HR1 cells do not express EBNA-2 and consequently express a very low level of LMP-1 due to the lack of EBNA-2 transactivation of the LMP-1 promoter (1, 18, 61, 64). Akata, Kem I, Daudi, SFC4, and IB4 cells are all EBV-positive cell lines. LCL1 to LCL7 are EBV-transformed B-cell lines collected from the laboratories of K. Izumi and L. Hutt-Fletcher. These cells were maintained in RPMI 1640 plus 10% fetal bovine serum (FBS). Electroporation (320 V; 925 μF) was used for the transfection of DG75 cells as described previously (74, 75, 77). One day after transfection, the cells were used for the isolation of CD4-positive cells with the use of Dynabeads CD4 (Dynal Inc.). Primary B-cell isolation was done as described previously with the use of CD19 antibody-conjugated magnetic beads (Dynal Inc.) (79).

Transfection of IB4 cells. The transfection of IB4 cells was achieved by using an Amaza Nucleofector device. Cells (1 × 10⁶) were transfected with 5 μg of DNA in solution B and program U20. Transfected cells were immediately put into 12-well plates with RPMI medium plus FBS. After transfection by use of an Amaza transfection apparatus, approximately 50% of cells were dead. The growth rates of cells after transfection were slower than those of untransfected cells regardless of the plasmid used. Approximately 70% of the remaining live cells contained transfected plasmids with the protocol. One day later, live cells were isolated by Ficoll-Paque Plus (GE Healthcare) according to the manufacturer’s recommendations. The live cells were counted and dispensed in culture flask at 3.5 × 10⁵ cells/ml: this was counted as day 1 after transfection. A small portion of cells were stained daily with trypan blue, and live cells were counted using a hemocytometer. A paired Student’s t test was used for statistical analyses.

Cell proliferation assay and Western blot analysis. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) growth assay was used to measure the cell proliferation rate using a CellTiter 96 nonradioactive cell proliferation assay kit (Promega) according to the manufacturer’s recommendations. Briefly, on day 3, a total 5 × 10⁵ live cells were collected and placed into 85 μl RPMI medium plus 10% FBS in a 96-well plate. Fifteen microliters of dye solution was added to each well. The cells were incubated in 37°C for 4 h. One hundred microliters of solubilization solution/stop mix was added and incubated for another hour. The absorbance at a wavelength of 562 nm was recorded using a 96-well plate reader. The separation of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were carried out according to standard protocol as described previously (74, 78).

DNA fragmentation assay. On day 3, the same volumes of cells were pelleted, and DNA isolation was performed under conditions described previously (28). The isolated DNA was separated on an agarose gel and photographed. This method preferentially isolates small DNA molecules.

Clinical samples and immunohistochemical and statistical analysis. A total of 27 primary CNS lymphomas were collected form the archives of the Pathology Institute, Lausanne, Switzerland, and the Neuropathology Core, Department of

| Table 1: Expression of IRF-4 in CNS lymphoma specimens |
|-----------------|-----------------|-----------------|-----------------|
| Case | Age of patient (yr) | CNS disease | Gender | Expression level |
| 1 | 47 | AIDS | Female | +++ + |
| 2 | 38 | AIDS | Male | ++ + |
| 3 | 58 | AIDS | Female | + + |
| 4 | 62 | AIDS | Female | + + |
| 5 | 35 | AIDS | Male | ++ + |
| 6 | 62 | AIDS | Male | + ++ |
| 7 | 38 | AIDS | Male | ++ + |
| 8 | 69 | AIDS | Female | ++ + |
| 9 | 27 | AIDS | Male | ++ + |
| 10 | 42 | AIDS | Male | ++ + |
| 11 | 39 | AIDS | Male | + + |
| 12 | 49 | AIDS, CMV | Male | + + |
| 13 | 77 | AIDS, toxoplasmosis | Female | + + |
| 14 | 80 | Alzheimer’s disease | Male | + + |
| 15 | 63 | Schizophrenia | Female | + + |
| 16 | 60 | NR | Male | +++ + |
| 17 | 82 | NR | Male | + + |
| 18 | 69 | NR | Male | +++ + |
| 19 | 67 | NR | Male | + + |
| 20 | 79 | NR | Male | ++ + |
| 21 | 76 | NR | Male | + + |
| 22 | 72 | NR | Female | + + |
| 23 | 78 | NR | Female | + + |
| 24 | 65 | NR | Female | + + |
| 25 | 86 | NR | Male | + + |
| 26 | 67 | NR | Female | + + |
| 27 | 69 | NR | Female | + + |

*The diagnosis of primary CNS lymphoma was based on the World Health Organization classification of brain tumors. CMV, cytomegalovirus; NR, no report on the clinical history. LMP-1 is used to represent the status of EBV; the data for LMP-1 were described previously (78) and are presented here for the benefit of the readers. “−”, negative reactivity; “+”, 1 to 30% cell positivity; “++”, 31 to 60% cell positivity; “+++”, >60% cell positivity. The association between expression levels of LMP-1 and IRF-4 is statistically significant (R = 0.479; F = 7.444; P = 0.011). Multiple regression analysis was done with Microsoft Excel.*

RESULTS

IRF-4 is induced during EBV transformation processes of human B cells in vitro. IRFs are implicated in the pathogenesis of human cancers (26, 59, 60, 78). IRF-4 was identified as being an LMP-1-inducible gene by microarray techniques (7). Since IRF-7 has been shown to be a potential factor in EBV transformation processes (78), we examined if IRF-4 is potentially involved in EBV transformation processes. Primary B cells were isolated from fresh blood, and the expression levels of IRF-4 for primary B cells and those for EBV-transformed cells (LCLs) were compared. As shown in Fig. 1A, high levels of IRF-4 expression were detected in EBV-transformed cells. In addition, we have shown previously with the same cell lysate that EBV-transformed cells express high levels of IRF-7 and
CD4 antibody-conjugated magnetic beads. As shown in Fig. 1, expression blotting after selection of the transfected cells by use of DG75 cells, and the levels of IRF-4 were determined by Western blotting. Lysates from primary B cells and EBV-transformed B cells were separated by SDS-PAGE. The expression levels of IRF-4 and tubulin were determined by Western blotting. The same membrane was stripped and reprobed with other antibodies. (B) IRF-4 is highly expressed in cells with EBV type III latency. Cell lysates from the indicated cell lines were separated by SDS-PAGE. The expression levels of IRF-4, LMP-1, EBNA-2, and tubulin proteins were determined by Western blotting. The images in the same box indicate that they are derived from the same membrane. The identities of proteins are shown.

Expression of IRF-4 is associated with EBV type III latency. To address the mechanism of IRF-4 induction during EBV transformation, we examined if IRF-4 expression is associated with EBV type III latency because type III latency is established during in vitro transformation. Sav I and Sav III (44, 76), Jijoye and P3HR1(2), and BL41 and BL41-EBV (10) are three paired cell lines (see Materials and Methods for details). As shown in Fig. 1B, IRF-4 was expressed at high levels in type III cell lines. The apparent molecular weight of EBNA2 is smaller in Jijoye cells, which is due to the differences in the EBNA2 gene in EBVs used to generate these cell lines (12).

We also examined IRF-4 expression in other latently EBV-infected cell lines. Daudi cells, like P3HR1 cells, lack EBNA2 in the EBV genome and have a very low level of LMP-1 expression. Akata and Kem I cells are type I latency BL lines. All these lines express low levels of IRF-4. However, SFC4, an EBV-transformed LCL with type III latency, expresses high levels of IRF-4 (data not shown). All these data indicate that the expression of IRF-4 is associated with EBV type III latency.

LMP-1 stimulates the expression of the IRF-4 protein. Because EBNA2 is the primary inducer of LMP-1 mRNA (1, 18, 61, 64), and because of the consistent association between IRF-4 and type III latency (Fig. 1), it is possible that EBNA2 and/or LMP-1 is responsible for the induction of IRF-4. EBV-negative DG75 cells were used to determine which viral gene could directly induce the expression of IRF-4. LMP-1 or EBNA2 and a CD4 expression plasmid were transfected into DG75 cells, and the levels of IRF-4 were determined by Western blotting after selection of the transfected cells by use of CD4 antibody-conjugated magnetic beads. As shown in Fig. 2A, LMP-1 expression causes a marked increase in IRF-4 protein levels in DG75 cells; however, EBNA2 seems to have a limited effect on the expression of IRF-4. The same results were obtained with BL41 cells, an EBV-negative BL line (data not shown). Therefore, LMP-1 appears to directly induce IRF-4 in type III latency. However, EBNA2 could help induce the expression of IRF-4 indirectly via the induction of LMP-1 in EBV-infected cells.

Signaling derived from LMP-1 is required for the induction of IRF-4. LMP-1 is an integral membrane protein with two C-terminal activating regions (CTARs) that have been shown to initiate signaling processes including the activation of NF-κB and IRF-7. LMP-DM is a mutant of LMP-1 in both CTARs that fails to activate NF-κB and IRF-7 (77). LMP-1 or LMP-DM and a CD4 expression plasmid were transfected into DG75 cells, and the expression of IRF-4 was examined. As shown in Fig. 2B, while wild-type LMP-1 strongly induced IRF-4, LMP-DM failed to induce IRF-4. Thus, signaling from LMP-1 CTARs is required for the induction of IRF-4.

Since NF-κB is a critical mediator of the LMP-1 signaling pathway (20, 54), we examined the involvement of NF-κB in the induction of IRF-4 by LMP-1. LMP-1 alone induced high levels of IRF-4 in DG75 cells. However, the coexpression of LMP-1 and IκB, a repressor of NF-κB, abolished the expression of IRF-4 (Fig. 2B). IκB expression was confirmed because the LMP-1-mediated activation of an NF-κB reporter gene was inhibited (data not shown). All these data suggest that NF-κB activation is required for the LMP-1-mediated induction of IRF-4.

Knockdown of IRF-4 expression inhibits the growth of EBV-transformed cells. We selected IRF-4 target sequences by comparing all the human IRF sequences and identified three unique sequences of IRF-4. Corresponding oligonucleotides were synthesized and cloned into an shRNA-expressing vector. The three different IRF-4 shRNAs (shIRF4) could in-
IRF-4 is involved in the growth control of EBV-transformed cells. Therefore, these data suggest that shIRF4 caused a severe slowdown in cell growth in medium containing LMP-1, EBNA-2, IRF-1, IRF-2, IRF-3, tubulin, and GAPDH proteins. Each point represents the number of live cells (means ± standard deviations) from three different counting. One representative from two independent experiments is shown. (C) IFN treatment did not affect the growth of IB4 cells. IFN-α (100 U/ml) was used to treat IB4 cells. Trypan blue was used to stain the cells, and live cells were counted. Each point represents the number of live cells (means ± standard deviations) from three different counting. One representative from two independent experiments is shown.

Knockdown of IRF-4 expression reduces the proliferation rate and enhances apoptosis. IRF-4 has oncogenic potential and may also prevent apoptotic processes (36). The IRF-4 knockdown-mediated cell growth inhibition may reflect a decrease in the rate of cell proliferation and/or an increase in the level of apoptosis.

To assess the proliferation rate, the same numbers of cells were plated into a 96-well plate, and MTT assays were carried out at day 3 of transfection. As shown in Fig. 4A, cells expressing IRF-4 shRNA have lower metabolic activities than cells expressing shRNA for luciferase (shLuc). Thus, shIRF4-expressing cells may have a lower proliferation rate than control cells based on the MTT assay.

To examine if apoptosis contributed to the reduced growth rates of shIRF4-expressing cells, a DNA fragmentation assay was performed. Apoptosis, or programmed cell death, is involved in the regulation of cell number under a wide variety of pathophysiological conditions (13, 45, 51, 56). One of the hallmark features of apoptosis is DNA fragmentation. Cellular DNA was isolated from shLuc- or shIRF4-expressing cells. As shown in Fig. 4B, shIRF4-expressing cells have a high level of fragmented DNA molecules. During apoptosis, poly(ADP-ribose) polymerase (PARP) is cleaved (30, 70). As shown in Fig. 4C, the IRF-4 knockdown resulted in PARP cleavage. Interestingly, an additional 50-kDa PARP fragment was detected (Fig. 4C), which might suggest that necrosis had occurred (53). We also detected the cleavage of caspase-3 in shIRF4-expressing cells (data not shown). All these data suggest that the knockdown of IRF-4 resulted in decreased proliferation and enhanced apoptosis.

IRF-4 is associated with LMP-1 in primary CNS lymphomas in vivo. Primary CNS lymphoma occurs almost exclusively in patients with AIDS. EBV is believed to be an important etiological agent of the disease (21, 38, 49, 52, 57). We have 27 human primary CNS lymphoma specimens available (78). The tumors were characterized according to the latest World Health Organization classification of brain tumors (34). These patients had been diagnosed with primary CNS lymphoma without the involvement of any other organ or tissue. Histologically, the tumors were characterized by abundant homogeneous neoplastic lymphocytes, located predominantly within the Virchow-Robin space in a concentric pattern. As shown in Fig. 5, the neoplastic cells had infiltrated the brain parenchyma in the majority of the cases. Pan-B (CD20) and pan-T (CD3) markers were used to determine the origin of the tumors. All of the tumors studied demonstrated a cytoplasmic expression of CD20 and no CD3 expression, indicating the B-cell origin of the lymphoma cells. The EBV status was determined by the expression of LMP-1 (78). IRF-4 is apparently localized in the nuclei predominantly (Fig. 5). IRF-4 was detected in 12 of the total 27 tumor samples examined (44.4%), and all IRF-4-positive specimens also expressed LMP-1 (12/12 [100%]) (Table 1). The association between the expression levels of LMP-1 and IRF-4 was highly significant (55).

A battery of EBV-transformed cell lines was screened for cells with good transfection efficiencies. IB4, which is a prototypical cell line transformed by EBV in vitro (8, 9, 11, 16, 22, 25), was found to have a relatively high level of efficiency of transfection with Amaxa’s technology. The transfection of IB4 cells with shIRF4 expression plasmids dramatically reduced the expression of IRF-4, whereas the expression levels of LMP-1, EBNA-2, IRF-1, IRF-2, IRF-3, tubulin, and GAPDH were unaffected (Fig. 3A). In addition, the level of expression of IRF-4 was not reduced by shIRF4 (data not shown). These data suggest that shIRF4 specifically reduced the expression of the IRF-4 protein.

The transfected cells were placed into culture dishes, and live-cell numbers were measured on a daily basis. As shown in Fig. 3B, shIRF4 significantly inhibited cell growth. shIRF4 also caused a severe slowdown in cell growth in medium containing less serum (data not shown). Therefore, these data suggest that IRF-4 is involved in the growth control of EBV-transformed cells.

Certain shRNAs may activate type I IFN pathways (55), which in turn may inhibit cell growth. The effect of IFN-α on the growth property of IB4 cells was examined. As shown in Fig. 3C, IFN had limited effects on cell growth, as expected (4, 29). The IB4 cells were responsive to IFN as determined by the activation of STAT-1 (72; data not shown). Thus, the shIRF4-mediated inhibition of cell growth was not apparently related to the potential activation of the IFN pathway.

FIG. 3. Knockdown of IRF-4 inhibits the growth of EBV-transformed cells. (A) shIRF4 specifically reduced the expression of endogenous IRF-4. An EBV-transformed cell line (IB4) was transfected with shLuc or shIRF4 by use of an Amaxa Nucleofector device. Three shIRF4 plasmids that target different regions of IRF-4 were used together to knock down IRF-4. The expression levels of IRF-4, LMP-1, EBNA-2, IRF-1, IRF-2, IRF-3, tubulin, and GAPDH were examined by Western blots. The identities of the proteins are shown. The images in the same box indicate that they are derived from the same membrane. (B) Knockdown of IRF-4 inhibits the growth of EBV-transformed cells. One day after transfection, live cells were isolated through Ficoll-Paque Plus and seeded. At the indicated days after transfection, surviving cells were enumerated by trypan blue exclusion. Each point represents the number of live cells (means ± standard deviations) from three different counts. One representative from five independent experiments is shown. (C) IFN treatment did not affect growth of IB4 cells. IFN-α (100 U/ml) was used to treat IB4 cells. Trypan blue was used to stain the cells, and live cells were counted. Each point represents the number of live cells (means ± standard deviations) from three different counting. One representative from two independent experiments is shown.

Knockdown of IRF-4 inhibits the growth of IB4 cells. IFN-α (100 U/ml) was used to treat IB4 cells. Trypan blue was used to stain the cells, and live cells were counted. Each point represents the number of live cells (means ± standard deviations) from three different counting. One representative from two independent experiments is shown.

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and IRF-4 was statistically significant ($R = 0.479; F = 7.444; P = 0.011$) (Table 1). These data strongly suggest that IRF-4 is associated with EBV infection in vivo and LMP-1 expression in particular in these primary CNS lymphomas.

DISCUSSION

EBV is able to transform primary human B cells in vitro. LMP-1 is the principal oncoprotein required for EBV transformation. Because transformation in vitro takes place rather rapidly and has a low probability of accumulating genetic mutations, at least initially, LMP-1 must contribute to cellular transformation by altering the expression and activity of cellular genes that are involved in oncogenesis.

In this report, we have provided evidence that cellular IRF-4 may be a key mediator for EBV transformation. First, IRF-4 expression was induced during EBV transformation in vitro and is correlated with LMP-1 expression from EBV-infected cells (Fig. 1). Second, IRF-4 expression was detected in a significant number of primary CNS lymphoma specimens and associated with the expression of LMP-1 in vivo in primary CNS lymphomas (Fig. 5 and Table 1). Third, LMP-1 induced the expression of IRF-4 proteins in transient assays. Furthermore, we have shown that NF-kB, which is a downstream target of the LMP-1 signaling pathway, is required for LMP-1 to induce IRF-4 (Fig. 2). Fourth, the knockdown of IRF-4 by shRNA in EBV-transformed cells resulted in the inhibition of cellular growth, apparently due to the collective effects of a reduced proliferation rate as well as increased apoptosis (Fig. 3 and 4). Because shIRF4 specifically down-regulates IRF-4, but not IRF-1, -2, -3, and -7 (Fig. 3A and data not shown), the

FIG. 4. Knockdown of IRF-4 reduces the proliferation rate but enhances apoptosis. (A) Reduction of proliferation rate in IRF-4 knockdown cells. The transfection of IB4 cells were achieved by using an Amaxa Nucleofector device. Three days later, equal amounts of live cells were placed into a 96-well plate, and MTT assays were used for the detection of the proliferation rate. Relative proliferation rates are shown. (B) DNA fragmentation in shIRF-4-transfected cells. Total cellular DNAs were isolated on day 3 and separated in agarose gels. The sizes of the DNA markers are as shown on the left in base-pairs. (C) PARP cleavage in IRF-4 knockdown cells. Lysates from transfected cells at day 3 were used for Western blot analysis with PARP, IRF-4, and GAPDH antibodies. The molecular masses (kDa) of PARP fragments are indicated in parenthesis. The identities of the proteins are as shown.

FIG. 5. Histological and immunohistochemical characterization of IRF-4 in CNS lymphomas. (A) Hematoxylin and eosin staining of a CNS lymphoma demonstrated the perivascular concentric accumulation of neoplastic cells. (B) Immunohistochemistry for the pan-B-cell marker CD20 highlighted the neoplastic lymphocytes in the Virchow-Robin space and confirmed their B-cell origin. (C) The EBV LMP-1 was robustly expressed in the cytoplasm of neoplastic lymphocytes in both the perivascular space and the brain parenchyma. (D) The expression of IRF-4 was detected in numerous neoplastic lymphocytes.
potential off-target effects of shIRF4 may be minimal. Fifth, the growth of IB4 cells was not affected in the presence of IFN (Fig. 3C), which is in agreement with data from previous reports (4, 29). In addition, we observed no IFN production due to the expression of shIRF4 by enzyme-linked immunosorbent assays (data not shown). Moreover, EBV EBNA2, which is not affected by shIRF4 (Fig. 3A), could alleviate the IFN-mediated growth arrest (29). Thus, the activation of the IFN pathway is not apparently involved in the alteration of growth phenotypes by IRF-4 knockdown. All these data collectively suggest that IRF-4 is involved in maintaining the growth phenotypes of EBV-transformed cells in vitro.

It is known that IRF-4 interacts with other cellular proteins for its proper functions (39). IRF-4 alone is apparently not sufficient for oncogenesis in the transgenic mouse model (50), suggesting that an additional factor(s) is required for the oncogenic activity of IRF-4 in vivo. We reason that EBV transformation may require functional interactions among IRF-4 and other viral and cellular factors. The depletion of IRF-4 might impair the transformation processes despite the physical presence of other oncogenic genes, such as IRF-7 and LMP-1. However, the direct targets of IRF-4 during EBV-induced transformation are currently unknown.

IRF-2, -4, and -7 are the three IRF members with oncogenic potential. IRF-7, similar to IRF-4, is associated with EBV transformation in vitro and in vivo. IRF-2 is apparently not associated with the EBV transformation process in vitro (78); however, IRF-2 is associated with type III latency and may negatively regulate an important viral latency promoter, Qp (73). Interestingly, IRF-5, which is likely a tumor suppressor (5, 23, 24, 43, 68), is highly expressed in EBV-transformed cells and, together with IRF-4, may be involved in the EBV-mediated regulation of Toll-like receptor 7 activities (40). Clearly, IRFs are important for EBV latency and transformation, potentially by targeting different or overlapping signaling pathways.

IRF-4 may play a role in EBV-associated tumors in vivo. IRF-4 expression was detected in a significant number of clinical samples of primary CNS lymphoma specimens. All IRF-4-positive specimens also expressed EBV LMP-1 (100%). In these LMP-1-negative specimens, no IRF-4 was detected (100%). However, 12/24 (50%) LMP-1-positive specimens had no detectable IRF-4. For those LMP-1-positive, IRF-4-negative specimens, the expression of LMP-1 was apparently lower: 9/12 (75%) specimens expressed low levels of LMP-1 (1 to 30% cell-positive reactivity). However, in those LMP-1-positive, IRF-4-negative specimens, LMP-1 expression was apparently higher: 9/12 (75%) specimens expressed LMP-1 at high levels (>30% cell-positive reactivity) (Table 1). We have found that the IRF-4 antibody used in this study had a moderate sensitivity for immunostaining (data not shown). Thus, the numbers of IRF-4-positive CNS lymphoma specimens might be higher than what was detected. Nevertheless, there is a statistically significant association between expression levels of LMP-1 and those of IRF-4 in these samples (P = 0.011) (Table 1). These data suggest that IRF-4 may be associated with LMP-1 in vivo. CNS lymphoma cells are phenotypically similar to EBV-transformed LCLs in vitro, and EBV is believed to be an etiological agent of AIDS-associated CNS lymphoma in vivo. Because of the critical functions of IRF-4 in the growth control of EBV-transformed cells in vitro (Fig. 3 and 4), it is likely that IRF-4 may play a similar role in vivo in the pathogenesis of CNS lymphoma.

In summary, this report has provided new insights into the EBV transformation process and suggests that IRF-4 may be involved in EBV transformation processes both in vitro and in vivo. In addition, IRF-4 might be a useful target in the therapy of EBV-mediated cellular proliferation.

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