Stimulation of c-Myc Transcriptional Activity by vIRF-3 of Kaposi Sarcoma-associated Herpesvirus*

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Kaposi sarcoma-associated herpesvirus is associated with two lymphoproliferative disorders, primary effusion lymphoma (PEL) and Castleman disease. In PEL, Kaposi sarcoma-associated herpesvirus is present in a latent form expressing only few viral genes. Among them is a viral homologue of cellular interferon regulatory factors, vIRF-3. To study the role of vIRF-3 in PEL lymphomagenesis, we analyzed the interaction of vIRF-3 with cellular proteins. Using yeast two-hybrid screen, we detected the association between vIRF-3 and c-Myc suppressor, MM-1α. The vIRF-3 and MM-1α interaction was also demonstrated by glutathione S-transferase pulldown assay and co-immunoprecipitation of endogenous MM-1α and vIRF-3 in PEL-derived cell lines. Overexpression of vIRF-3 enhanced the c-Myc-dependent transcription of the gene cdk4. Addressing the molecular mechanism of the vIRF-3-mediated stimulation, we demonstrated that the association between MM-1α and c-Myc was inhibited by vIRF-3. Furthermore, the recruitment of vIRF-3 to the cdk4 promoter and the elevated levels of the histone H3 acetylation suggest the direct involvement of vIRF-3 in the activation of c-Myc-mediated transcription. These findings indicate that vIRF-3 can effectively stimulate c-Myc function in PEL cells and consequently contribute to de-regulation of B-cell growth and differentiation.

Kaposi sarcoma-associated herpes virus (KSHV) is a member of the γ herpes virus family that is genetically similar to Epstein-Barr virus and monkey herpes virus Saimiri (1). Sequence analysis of KSHV genome revealed the presence of ~80 open reading frames (ORFs) of which a number of ORFs shows homology to cellular genes that regulate cell growth, immune functions, inflammation, and apoptosis (2–5). These include a cluster of four ORFs with homology to the cellular transcription factors of the interferon regulatory factor (IRF) family (6, 7). One of them, viral interferon regulatory factor-3 (vIRF-3, also referred to as LANA2) (8–10), is a multifunctional nuclear protein constitutively expressed in KSHV-positive primary effusion lymphoma (PEL) cells and Castleman disease tumors, whereas it is not detected in Kaposi sarcoma spindle cells (8, 10). The vIRF-3 protein binds to cellular IRF-3 and IRF-7 and to the transcription co-activators, CREB-binding protein/p300, and modulates IRF-3/IRF-7-mediated transcription of Type I interferon genes (9). Furthermore, interaction of vIRF-3 with p53 results in inhibition of p53 transcriptional activation and p53-induced apoptosis (10). Inhibition of the 1kB kinase kinase activity, and down-regulation of the NFκB-dependent transcription by vIRF-3 have also been reported (11). Recently, vIRF-3 was shown to interact with 14-3-3 proteins and inhibit FOXO3a transcription factor, which may contribute to cell cycle de-regulation (12).

KSHV-positive PEL is an aggressive B-cell lymphoma typically growing as lymphomatous effusions in the body cavities without a contiguous tumor mass. In addition to KSHV, some PEL cells also carry the Epstein-Barr virus genome, but are devoid of genetic lesions of c-myc, bcl-2, and p53 (13). Cell lines established from PEL continuously express only six viral genes, vFLIP (ORF71), vCYC (ORF72), latency-associated nuclear antigen-1, LANA (ORF73), Kaposin (K12) (14–17), vIRF-3/ LANa2, and short ORF K11.1 encoding vIRF-2 (8, 10, 18).

How KSHV contributes to the establishment of the KSHV-associated neoplasia remains to be determined. Proliferation of PEL cells in culture was found to depend on the autocrine production of KSHV-encoded vIL-6, expressed during lytic infection (19). However, because the expression of KSHV in PEL cells is latent, it is assumed that latent genes rather than the genes expressed during the lytic infection may contribute to leukemogenesis. Two of these latent genes, vFLIP and Kaposin, have transforming potential in vitro (20, 21); however, they were unable to induce tumors in vivo in transgenic mice. The expression of vGPCR was sufficient to induce endothelial tumors resembling Kaposi sarcoma (22, 23) but did not induce B-cell lymphomas.

Although it has been accepted that lymphomagenesis is a multistep transformation process, a number of genetic changes and infection agents contribute to B-cell lymphoproliferative disorders. c-myc is a proto-oncogene that has been implicated

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‡The abbreviations used are: KSHV, Kaposi sarcoma-associated herpesvirus; vIRF-3, viral interferon regulatory factor-3; LANA2, latency-associated nuclear antigen 2; MM-1, Myc modulator-1; HEK293, human embryonic kidney cells 293; GST, glutathione S-transferase; FL, full length; MBS, c-Myc binding site; ORF, open reading frame; IRF, interferon regulatory factor; PEL, primary effusion lymphoma; CREB, cAMP-response element-binding protein; aa, amino acid; HA, hemagglutinin; ChIP, chromatin immunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; pITB, retinoblastoma protein; X-α-gal, 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside.

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in controlling cellular growth, proliferation, and cell survival (24). It also plays a critical role in the development of B-cell lymphomas (25–27). Overexpression of c-myc, as a consequence of a reciprocal chromosomal exchange involving the immunoglobulin loci, can be seen in Epstein-Barr virus-positive B-cell lymphomas. Expression of c-myc in transgenic mice results in the formation of pre-B-cell lymphoma (28) and immature CD4+ and CD8+ T-cell lymphoma (29). Thus, overexpression of c-myc may have an important role in lymphoid cell neoplasia. The ability of c-Myc to promote proliferation through cell cycle re-entry seems critical to its oncogenic function. Constitutive expression of c-myc reduces the growth factor requirement, prevents growth arrest, and blocks cellular differentiation (30, 31). The c-myc gene encodes a transcription factor of the basic-helix-loop-helix-leucine zipper family. c-Myc dimerization with another basic-helix-loop-helix-leucine zipper protein, Max, is required for its binding to the specific DNA sequence CACGTG (E-box) and activation of transcription (32, 33). Two domains of c-Myc are crucial for its biological activities. The N-terminal domain consists of the transcriptional activation domain, whereas the C-terminal domain mediates DNA binding to promoters of target genes. In recent years, many new C-terminal domain- and N-terminal domain-interacting proteins have been identified (34, 35).

The transcriptional activity of Myc can be repressed by FoxO proteins (36), and by a novel c-Myc repressor, Myc modulator-1 (MM-1). MM-1 binds to the N-terminal domain of c-Myc and suppresses its E-box-dependent transcriptional activity (37). A mutation of Ala to Arg at amino acid 157 in MM-1, which is frequently observed in patients with leukemia and lymphoma, abrogated the MM-1 suppression activity of c-Myc (38). MM-1 cDNA was originally identified as a fusion gene described previously (8). Expression plasmid c-myc-HA and cdk4 reporter constructs, wtMBS1–4, mutMBS1–4, and mutMBS3 + 4, were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). The 4XE-box-luc reporter plasmid was kindly provided by Dr. Hiroyoshi Ariga (Hokkaido University, Sapporo, Japan).

**Stimulation of c-Myc Activity by vIRF-3**

For the yeast-two-hybrid screening, the vIRF-3-C′/pAS2 was cloned by inserting the C-terminal part of vIRF-3 (aa 254–566) between the EcoRI and BamHI sites of pAS2–1 (Clontech Laboratories, Inc., Palo Alto, CA). Full-length vIRF-3 (vIRF-3-FL; aa 1–566), vIRF-3-N′ (aa 1–254), vIRF-3-C′ (aa 254–566) and vIRF-3-GST were described previously (8). Expression plasmid c-myc-HA and cdk4 reporter constructs, wtMBS1–4, mutMBS1–4, and mutMBS3 + 4, were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). The 4XE-box-luc reporter plasmid was kindly provided by Dr. Hiroyoshi Ariga (Hokkaido University, Sapporo, Japan). The T7-MM-1α expression plasmid was cloned by reverse transcription-PCR amplification of MM-1α cDNA using RNA isolated from BCBL-1 cells. The 5′ primer carried a T7 tag sequence that was in-frame with the MM-1α open reading frame. The PCR product was subcloned into pcDNA3.1 vector (Invitrogen). GST-MM-1α-FL (aa 1–154), and its deletion constructs GST-MM-1α(1–125), (1–93)–(1–62), (1–30)–(1–62), and (63–154) were cloned by amplification of the corresponding region of MM-1α cDNA from the T7-MM-1α expression plasmid and subcloned into pGEX-4T vector (Amersham Biosciences). The fidelity of all constructs was verified on the ABI PRISM™ 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). The following antibodies were used: polyclonal antibodies against c-Myc (N262), p73, IRF-3, actin, and HA (Santa Cruz Biotechnology Inc., Santa Cruz, CA), T7 monoclonal antibodies (Novagen, EMD Chemicals Inc., San Diego, CA), acetylated-histone 3 antibodies (Upstate Millipore, Billerica, MA), and antibodies against GST (Amersham Biosciences). Production and purification of polyclonal antibodies against vIRF-3 was described previously (9). The MM-1α monoclonal antibodies were kindly provided by Dr. Hiroyoshi Ariga.

** Yeast Two-hybrid Screen** — The MATCHMAKER Two-hybrid System 3 was purchased from Clontech Laboratories, Inc. The bait plasmid, vIRF-3-C′/pAS2, and the human leukocyte cDNA library (Clontech Laboratories, Inc.) were introduced into the Saccharomyces cerevisiae strain AH109 using the lithium acetate/heat shock procedure (41). The transformed yeast cells were plated on SD/-Ade/-His/-Leu/-Trp/X-α-gal medium and incubated for 1 week at 30 °C. The interacting cDNA clones were rescued from positive colonies. The sequences of positive cDNA clones were determined on ABI PRISM™ 377 automated DNA sequencer (Applied Biosystems) and analyzed using the BLAST program.

**Immunoprecipitation and Western Blot Analysis** — HEK293 cells were co-transfected with vIRF-3, c-myc-HA, and T7-MM-1α expression plasmids using the SuperFect transfection reagent (Qiagen). At 24 h post-transfection, the cells were lysed in co-immunoprecipitation buffer (20 mM HEPES (pH 7.9), 50 mM NaCl, 5 mM EDTA, 2 mM EGTA, 0.1% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.2 mM protease inhibitor mixture (Sigma Co., St. Louis, MO)). The protein extracts (400 μg) were then incubated with the respective antibodies at 4 °C for 1 h, and then 30 μl of protein A/G- Sepharose beads (Santa Cruz Biotechnology Inc.) was added, followed by overnight incubation at 4 °C. Immune complexes were extensively washed with co-immunoprecipitation buffers, and then eluted with SDS-PAGE sample buffer.
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Luciferase Assays—For luciferase assays with HEK293 cells, the cells were seeded in 6-well tissue culture plates 12 h before transfection. Sub-confluent cells were transfected with equal amounts (1 μg) of luciferase reporter and vIRF-3, c-Myc-HA, or T7-MM-1α expression plasmids together with control plasmid pRL-SV40 (0.1 μg, Promega, Madison, WI) using SuperFect transfection reagent (Qiagen). Forty-eight hours after transfection the cells were lysed with the Reporter Lysis Buffer (Promega), and luciferase activity was measured in 20 μl of the lysate using Dual Luciferase Reporter Assay Kit (Promega) as recommended by the manufacturer. Each experiment was repeated three times. The Renilla luciferase activity levels were used to normalize the differences in the transfection efficiency. For luciferase assays with BCBL-1 cells, the procedure was identical except that transfections were done using the Nucleofector transfection device (Amaza Inc., Gaithersburg, MD).

GST Pulldown Assay—In vitro translated proteins were synthesized using the coupled TnT T7 transcription-translation system (Promega) according to the manufacturer’s instruction. GST fusion proteins (0.5 μg) bound to glutathione-Sepharose beads were incubated with 10 μl of the reaction mixture consisting of in vitro translated proteins in 250 μl of binding buffer (10 mM Tris (pH 7.6), 100 mM NaCl, 0.1 mM EDTA (pH 8.0), 1 mM dithiothreitol, 5 mM MgCl₂, 0.05% Nonidet P-40, 8% glycerol, 0.2 mM protease inhibitor mixture (Sigma)) at 4 °C for 90 min. After three 10-min washes with binding buffer, the proteins bound to the beads were resolved by SDS-PAGE and detected by Western blotting with specific antibodies. For the control of quality and equal loading of GST fusion proteins, the Western blot was re-hybridized with GST antibodies (Amer- sham Biosciences).

Oligonucleotide Pulldown Assay—The DNA pulldown assay was done as previously described (42). Briefly, double-stranded oligomers corresponding to the MBS4 in the human cdk4 promoter region (5′−CCCTCGCGCCATGGGG- TGGCGGTTCAGTGCAGAAGTCCG-3′), and tetramerized E-box sequences, 4XE-box (5′−CCCTCGCGCCATGGGGTCGCGGTTCAGTGCAGAAGTCCG−3′; E-boxes are underlined), were synthesized and biotin-labeled at the 5′ end of the sense strand and coupled with streptavidin magnetic beads (Dynal, Invitrogen). Whole cell lysates (350 μg) were then incubated with the DNA bound to magnetic beads for 3 h at 4 °C. After extensive washing, the bound proteins were resolved by SDS-PAGE and analyzed by Western blot.

Chromatin Immunoprecipitation—The assay was performed using a chromatin immunoprecipitation assay kit (Upstate Millipore) following the manufacturer’s instructions. Briefly, for the endogenous cdk4 promoter studies, BCBL-1 or HEK293 cells (10⁷) were transfected with an empty vector, pcDNA3.1 (control), c-myc-HA-, or vIRF-3 expression plasmids. At 24 h post-transfection, the proteins bound to DNA were cross-linked in the presence of 1% formaldehyde; cells were resuspended in the SDS lysis buffer, followed by sonication. After pre-clearing with salmon sperm DNA/Protein A-agarose (50% slurry), the protein extracts were subjected to immunoprecipitation with antibodies against c-Myc, vIRF-3, or acetylated H3. Immunoprecipitation with antibodies against p73 or IRF-3 was used as a negative control. Immunocomplexes were extensively washed, and the DNA was recovered by phenol/chloroform extraction and resuspended in 50 μl of 10 mm Tris-HCl (pH 8.5). Serial dilutions (1, 5, 10, and 20 μl) were used as template for PCR amplification to show that the response was in the linear range. Each experiment was repeated three times. PCR amplification was performed with the following cdk4-specific primers: cdk4-FWD, 5′-AGTGAGACCATCTGTCAGCCG-3′; cdk4-REV, 5′-GACGTTCTGGGCAGTGAC-3′. The samples were also amplified with GAPDH primers: GAPDH- FWD, 5′-CCAACCTTTCCGCTCTC-3′; GAPDH-REV, 5′-CAGCAGGCTGTTCAACTG-3′, which were used as controls (43).

RESULTS

Isolation of MM-1α as a vIRF-3-binding Protein in Yeast Two-hybrid Screen—In the search for one or more cellular proteins that may interact with vIRF-3, we employed a yeast two-hybrid system to screen a human leukocyte cDNA library. Initially, we used the full-length vIRF-3 (vIRF-3-FL) as a bait, but the control experiments showed that vIRF-3 may have a strong activation domain, because introduction of the vIRF-3-FL/pAS2 construct into Saccharomyces cerevisiae strain AH109 yielded numerous positive colonies on plates with SD/-Ade/-His/-Leu/-Trp/X-a-gal medium. Therefore, we used the plasmid, vIRF-3-C′/pAS2, encoding only the C-terminal half (aa 254–566) of the vIRF-3 protein. Co-transformation of AH109 yeast cells with the vIRF-3-C′/pAS2 bait and the human leukocyte cDNA library resulted in 42 positive colonies that grew on selection medium lacking Trp, Leu, His, and Ade. 22 of 42 His/Ade-positive transformants formed blue colonies. Plasmids rescued from these positive colonies were analyzed using restriction enzymes, and their inserts were sequenced. One clone, termed C19, contained partial cDNA for human MM-1, which has been previously shown to be a c-Myc-binding protein (44). The cDNA insert of this plasmid encoded the protein ranging from amino acids 17–167 and 4–167 of MM-1 and MM-1α, respectively (Fig. 1A). Because the C19-encoded protein was almost identical to MM-1α, we cloned a T7-tagged MM-1α cDNA from a KSHV-positive PEL cell line, BCBL-1, into pcDNA3.1 vector and used the construct for the experiments described in this study. The originally isolated MM-1 (44), which represents a fusion gene derived from sequences on chromosome 14 and 12, and the recently identified isoform MM-1α (39), share the same properties and functions. Both of them are nuclear proteins that exhibit a strong inhibitory effect on c-Myc-mediated transcription (39).

Specificity of the MM-1α-vIRF-3 Interaction In Vitro and in Vivo—To confirm the results of the yeast two-hybrid assay, we first examined the interaction of vIRF-3 with MM-1α in vitro by a GST pulldown assay and then in vivo by co-immunoprecipitation. The results in Fig. 1B showed that in vitro translated MM-1α bound strongly to the full-length GST-vIRF-3 fusion protein, whereas, it did not interact with GST alone. The in vivo interaction between ectopically expressed vIRF-3 and MM-1α in HEK293 cells was examined by co-immunoprecipitation. As
shown in Fig. 1C, T7-tagged MM-1α co-precipitated with transfected vIRF-3 protein. In addition, we detected the interaction between endogenously expressed vIRF-3 and MM-1α proteins in two PEL-derived cell lines, BCBL-1 and BC-3 (Fig. 1D). Co-immunoprecipitation in Daudi cells, which are KSHV-negative and do not express vIRF-3, was used as a negative control.

Identification of the MM-1α Interaction Domain—To determine which part of MM-1α protein interacted with vIRF-3, we constructed a series of MM-1α deletion mutants, which were expressed as GST fusion proteins (Fig. 2A). The mobility and purity of these recombinant fusion proteins is shown in Fig. 2B. When used in the GST pulldown assay, all GST-MM-1α deletion mutants were able to bind specifically to the in vitro translated full-length vIRF-3, except the shortest MM-1α-(1–30)-GST and GST alone (Fig. 2C). These results suggest that the MM-1α protein interacted with vIRF-3 through the binding domain that was located in the region between amino acids 31 and 62. To confirm the binding of this peptide to vIRF-3, we constructed the MM-1α (aa 31–62) and MM-1α (aa 63–154) fused to GST and analyzed their ability to interact with vIRF-3 in the GST pulldown assay. The results in Fig. 2D showed that in vitro translated vIRF-3 bound with the same intensities to the MM-1α-GST and MM-1α-(31–62)-GST protein, whereas it did not interact with either MM-1α-(63–154)-GST or GST alone. These data suggest that the interaction between vIRF-3 and MM-1α is direct and that the region between amino acids 31 and 62 of MM-1α protein is its primary interaction domain with vIRF-3.

Stimulation of c-Myc Transcriptional Activity by vIRF-3—The ability of c-Myc to promote cell cycle re-entry is in part due to its ability to induce transcription of cdk4 (45). The cdk4 gene promoter contains four putative c-Myc binding sites (MBS1–4). Mutation analysis of individual MBS elements suggested that MBS3 and MBS4 were particularly important in the transactivation of cdk4 promoter by c-Myc (45). To determine the effect of vIRF-3 on transcriptional activity of c-Myc, we transfected HEK293 cells with c-myc, vIRF-3, and MM-1α expression plasmids together with a reporter construct containing the luciferase gene linked to the wild-type cdk4 promoter, wtMBS1–4. As shown in Fig. 3A, both c-Myc and vIRF-3 activated the cdk4 promoter by 2.4-fold, whereas MM-1α suppressed its activity. In addition, co-transfection of vIRF-3 together with c-myc resulted in further stimulation of cdk4 promoter activity to 3.3-fold. Additional co-transfection of MM-1α did not decrease the vIRF-3-mediated stimulation of the cdk4 promoter, suggesting that vIRF-3 can effectively reverse the MM-1α-mediated suppression. Similar data were obtained with the reporter plasmid containing the synthetic tetramerized 4XE-box promoter linked to luciferase gene (44) (Fig. 3B). Co-transfection of 4XE-box reporter and c-myc expression plasmid into BCBL-1 cells resulted in 2.4-fold activation. Expression of vIRF-3 alone also slightly stimulated the 4XE-box promoter, whereas co-expression of c-myc and vIRF-3 further enhanced the c-Myc-mediated activation of 4XE-box promoter to 3.2-fold. It should be noted that BCBL-1 cells constitutively express endogenous vIRF-3, which may account for relatively low levels of enhancement of the c-Myc transcriptional activity by ectopic vIRF-3.

To confirm that activation of the cdk4 promoter by vIRF-3 was due to its stimulatory effect on c-Myc-mediated transcription, we employed two mutated cdk4 reporter constructs (45). In the first mutated construct, mutMBS3+4, two (MBS3 and -4) out of four E-box sequences in the cdk4 promoter were
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FIGURE 2. Analysis of the vIRF-3-MM-1α interaction. A, schematic representation of the MM-1α deletion mutants. B, Coomassie Blue staining of MM-1α deletion mutants. GST fusion proteins corresponding to different MM-1α deletion mutants were immobilized on glutathione-Sepharose beads and subjected to SDS-PAGE followed by Coomassie Blue staining. C, in vitro translated vIRF-3 full-length (vIRF-3-FL) was incubated with different MM-1α deletion mutants fused to GST and immobilized on glutathione-Sepharose beads. The bound proteins were eluted and resolved on 8% SDS-PAGE followed by Western blot with vIRF-3 antibodies. 10% of vIRF-3-FL protein input is shown in lane 1 (input). The Western blot was re-hybridized with GST antibodies to assess the quality and quantity of MM-1α-GST deletion mutants (bottom panel). Schematic representation of MM-1α-GST deletion mutants is shown in the bottom panel.

FIGURE 3. vIRF-3 stimulates the c-Myc transcriptional activity. A, effect of vIRF-3 on activation of cdk4 promoter. Human wild-type cdk4 reporter (wtMBS1–4) and pRL-SV40 plasmids were co-transfected into HEK293 cells with either an empty vector (pcDNA3) or c-myc, MM-1α, and vIRF-3 expressing plasmids. Luciferase activity was analyzed 48 h post-transfection as -fold activation relative to the basal level of reporter gene in the presence of control vector, pcDNA3. Results were normalized to Renilla luciferase activity. Error bars represent standard errors for three experiments. B, effect of vIRF-3 on c-Myc-mediated activation of 4X-E-box promoter. BCBL-1 cells were co-transfected with 4X-E-box reporter and pRL-SV40 plasmids together with either empty vector (pcDNA3) or c-myc- and vIRF-3-expressing plasmids. Luciferase activity and data analyses were performed as in A. Error bars represent standard errors for three experiments.

mutated. As shown in Fig. 4A, this mutated reporter responded only marginally to the c-Myc activation, and co-transfection of c-myc and vIRF-3 did not result in significant stimulation. In the second reporter, mutMBS1–4, all four E-box sequences in the cdk4 promoter were mutated. Co-transfection of this reporter together with c-myc completely abrogated transactivation, and the activity of this promoter was not stimulated by c-Myc and vIRF-3. Thus when compared with the activity of the wild-type cdk4 promoter (wtMBS1–4), the point mutations of two or all four MBS (mutMBS3+4 and mutMBS1–4) markedly diminished the activation by c-Myc or the c-Myc-vIRF-3 complex. These data suggest that c-Myc and vIRF-3 activate the cdk4 promoter in an E-box-dependent manner.

To determine whether the C-terminal part of vIRF-3, which binds to MM-1α, is sufficient to elicit the stimulatory effect on the transcriptional activity of c-Myc, we have compared the effects of vIRF-3-FL, -N, and -C on the c-Myc-mediated activation of the cdk4 promoter. The HEK293 cells were transfected with wild-type cdk4 (wtMBS1–4) reporter together with plasmids expressing c-myc and vIRF-3-FL, and its N- and C-terminal parts. As shown in Fig. 4B, only the full-length vIRF-3 was able to effectively stimulate transcriptional activity of c-Myc, whereas neither N- nor C-terminal parts of vIRF-3 showed any stimulatory effect. These data indicate that the binding of the C-terminal part of vIRF-3 to MM-1α is not sufficient to reverse the MM-1α-mediated suppression of the c-Myc transcriptional activity.

vIRF-3 Competes with MM-1α for Binding to c-Myc—To address the molecular mechanism by which vIRF-3 rescues the c-Myc-mediated transcription in the presence of MM-1α, we examined whether vIRF-3 can interfere with MM-1α and c-Myc interaction. The MM-1α and c-Myc expression plasmids were co-transfected together with
Expression plasmid. 24 h after transfection, cell lysates (400 μg) were co-transfected into HEK293 cells and analyzed by Western blot with vIRF-3 or HA antibodies (c-Myc). The relative levels of vIRF-3, MM-1α, and c-Myc in the lysates (40 μg) of transfected cells were estimated by Western blot (inputs 10%).

Increasing amounts of vIRF-3 bound to MM-1α increased. These data suggest that vIRF-3 competes with c-Myc for binding to MM-1α, thus releasing c-Myc from MM-1α-mediated suppression.

Analysis of the Complex Assembled on the Promoters of c-Myc-responsive Genes—To analyze the composition of the complex assembled on the wtcdk4 promoter or the c-Myc binding E-box sequences (4XE-box), we performed a series of DNA pulldown analyses in which oligonucleotides corresponding to either the MBS4 of human wild-type cdk4 promoter or tetramerized E-box sequence (4XE-box) were biotinylated and coupled to streptavidin-coated magnetic beads. The DNA-containing beads were incubated with protein extracts from HEK293 cells transfected either with vIRF-3, c-Myc, MM-1α expression plasmids or their combination. The vIRF-3, c-Myc, or MM-1α proteins that were specifically bound to the promoter DNA were then detected by Western blot. As shown in Fig. 6A, c-Myc effectively bound to the wtcdk4 promoter sequences (Fig. 6A, lanes 1–4). Moreover, vIRF-3 also associated with these oligonucleotides (Fig. 6A, lanes 2 and 4), indicating that either vIRF-3 itself bound to the promoter DNA or it was tethered to the promoter by c-Myc protein. Notably, there was no detectable binding of the MM-1α protein to the promoter DNA, suggesting that there was either no association between MM-1α and the cdk4 promoter or MM-1α bound to DNA very weakly and transiently. Interestingly, the binding of vIRF-3 to the promoter was decreased in the presence of MM-1α (Fig. 6A, lane 4). We further analyzed the assembly of the c-Myc-vIRF-3 complex at the wtcdk4 or 4XE-box promoters (Fig. 6B). In agreement with our previous experiment, both c-Myc and vIRF-3 bound effectively to wtcdk4 promoter as well as 4XE-box sequences. In the absence of ectopic c-myc expression (Fig. 6B, lanes 1 and 4), the recruitment of vIRF-3 to the promoter sequences was most likely due to the DNA-binding capacity of vIRF-3 itself, or through its association with endogenous c-Myc. Surprisingly, the co-expression of c-myc and vIRF-3 resulted in an increased binding of these two proteins to DNA (Fig. 6B, lanes 3 and 6). The observed differences in the binding of c-Myc and vIRF-3 to the wtcdk4 and 4XE-box-containing promoters were not a consequence of different levels of their expression in the analyzed protein extracts, because both vIRF-3 and c-myc were expressed effectively in transfected cells, and their relative levels were almost identical in all samples tested (Fig. 6B, 10% inputs). To determine whether c-Myc contributes to the DNA-binding capacity of vIRF-3, we analyzed the assembly of the
transcription complex on the wtcdk4 promoter in c-myc-null HO15 cells. The results of the DNA pulldown assay showed that, in the absence of endogenous c-myc, the association of vIRF-3 with the wtcdk4 promoter is very low (Fig. 6C, lane 2), but it is profoundly increased in TGR-1 cells that express endogenous c-Myc protein (Fig. 6C, lane 4). These data suggest that vIRF-3 may be recruited to the cdk4 promoter through its interaction with c-Myc and that the presence of vIRF-3 increases the ability of c-Myc to bind to the cdk4 promoter (Fig. 6C, lane 4).

The results of DNA pulldown assays indicated that vIRF-3 may directly associate with c-Myc in the cells. As shown in Fig. 6D, co-immunoprecipitated vIRF-3 strongly interacted with endogenous c-Myc in vIRF-3-transfected HEK293 cells. These data support the results of DNA pulldown assay and suggest that vIRF-3 is part of the transcription complex assembled on cdk4 or synthetic E-box-containing promoters. Furthermore, the vIRF-3/c-Myc heterodimer appears to have higher DNA-binding capacity than the c-Myc protein alone.

To further analyze the recruitment of the vIRF-3/c-Myc heterodimer to the cdk4 promoter in vivo, we employed the ChIP assay. The assembly of c-Myc and vIRF-3 on endogenous cdk4 promoter was analyzed in KSHV-positive BCBL-1 cells that were transfected with either the empty vector or the c-myc-expressing plasmid. The proteins were then cross-linked to DNA, and the DNA-protein complexes were precipitated with antibodies against c-Myc, vIRF-3, and p73 (negative control). The DNA in the precipitates was then amplified by PCR with primers specific for the endogenous cdk4. As shown in Fig. 7A (lane 1, left panel), in BCBL-1 cells, the association of the c-Myc/vIRF-3 heterodimer with the cdk4 promoter is constitutive. Furthermore, in cells overexpressing c-myc, the recruitment of vIRF-3 to the promoter is increased indicating that vIRF-3 is recruited to the promoter through its binding to c-Myc (Fig. 7A, lane 2, left panel). It should be noted that the relative levels of endogenous vIRF-3 protein were unchanged by ectopic c-Myc (Fig. 7B). Neither c-Myc nor vIRF-3 was found to be recruited to the control GAPDH promoter (Fig. 7A, right panel), indicating that these proteins are specifically recruited to the cdk4 promoter. We have also tested the binding of ectopic T7-MM-1α protein to the endogenous cdk4 promoter by ChIP assay, but we were unable to detect any association (data not shown). These results are in agreement with our DNA pulldown experiments and
Support the finding that most likely MM-1α is not directly associated with the cdk4 promoter.

To further determine whether vIFR-3 modulated the association of c-Myc with the cdk4 promoter in cells, we performed a similar ChIP experiment with HEK293 cells. Cells were transfected with either an empty vector or the vIFR-3-expressing plasmid. As shown in Fig. 8A (left panel), the recruitment of endogenous c-Myc protein to the cdk4 promoter is significantly increased in the presence of vIFR-3. Notably, the levels of acetylation of histone H3 at the cdk4 promoter are much higher in cells expressing vIFR-3 than in control cells, suggesting that vIFR-3 stimulates the transcription of cdk4 gene. Neither c-Myc nor vIFR-3 were recruited to the control GAPDH promoter (Fig. 8A, right panel), confirming the specificity of binding of these proteins to the cdk4 promoter. In addition the levels of histone H3 acetylation at the GAPDH promoter were not modulated by vIFR-3. It should be noted that the relative levels of endogenous c-Myc protein were unchanged by ectopic vIFR-3 as assayed by Western blot (Fig. 8B). Altogether, these data suggest that vIFR-3 stimulates the c-Myc-mediated transcription of cdk4 gene, which is reflected by strong binding of c-Myc/vIFR-3 heterodimer to E-boxes of cdk4 promoter.

**DISCUSSION**

In this study we show that in two PEL-derived cell lines latently infected with KSHV, vIFR-3 associates with the c-Myc repressor, MM-1α. As a result of direct binding of vIFR-3 to MM-1α, c-Myc is released from the MM-1α-mediated suppression and stimulates transcriptional activity of cdk4 promoter and possibly the promoters of other c-Myc-regulated genes. It was shown previously that the ability of c-Myc to stimulate cdk4 transcription results in the promotion of cell cycle re-entry. Additionally, there is strong evidence that the cdk4 gene plays a role in carcinogenesis. When complexed with cyclin D, cdk4 inhibits the activity of retinoblastoma protein (pRb) (46, 47), thus linking the function of c-Myc to the cdk4-cyclin D-pRb pathway. Overexpression of cdk4, together with activated Ras, is sufficient to induce neoplastic transformation (48, 49).

The cdk4 promoter contains four highly conserved E-box elements, which bind c-Myc and thus play a critical role in the c-Myc-mediated activation (45). Here we show that activation of cdk4 promoter by vIFR-3 is also E-box-dependent. Furthermore, vIFR-3 is recruited to the cdk4 promoter in KSHV-positive PEL-derived cell lines via its interaction with c-Myc. The association of vIFR-3 with c-Myc may increase the c-Myc stability or DNA-binding capacity, which is reflected by stronger binding of c-Myc to the cdk4 promoter in the presence of vIFR-3. However, stimulation of cdk4 transcription by c-Myc and c-Myc-vIFR-3 complexes results only in a relatively modest increase. These low levels of change correlate with previous observations that c-Myc is a weak transcription activator (50, 51). For the stimulatory effect on the c-Myc-mediated transcription, the full-length vIFR-3 protein is required. It is possible that, although the C terminus of vIFR-3 mediates binding to MM-1α, the N-terminal half of vIFR-3 may facilitate the association of vIFR-3 with c-Myc, or may serve as a bridge between...
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the c-Myc protein and other transcription co-factors and/or co-activators.

It is generally assumed that chromatin remodeling involves the bridging of the sequence-specific transcription factors and the basal transcription factors by the co-repressor or co-activator complex (52). MM-1α antagonizes the activation function of c-Myc by binding to c-Myc and recruiting the co-repressor complex consisting of TIF-1β, HDACs, and mSin3a (37, 44, 53). This ultimately results in the inhibition of c-Myc transcriptional activity. Here we demonstrated that vIRF-3 binds to MM-1α and releases c-Myc from MM-1α-mediated repression. Furthermore, vIRF-3 forms a heterodimer with c-Myc, which binds to the E-boxes in the cdk4 promoter and stimulates their transcription. The association of the c-Myc/vIRF-3 heterodimer with the cdk4 promoter is followed by increased levels of histone H3 acetylation, which is considered to be a general hallmark of active transcription. We have previously observed that vIRF-3 can act as a potent stimulator of IRF-3- and IRF-7-mediated transcriptional activation of Type I interferon (IFNA and IFNB) gene promoters (9). This stimulation was due to the association of vIRF-3 with IRF-3 and IRF-7 followed by the recruitment of CREB-binding protein/p300 to the transcription enhancerosome assembled on the promoters of IFNA/B genes (9). Thus, the possible involvement of histone acetyltransferases in the stimulation of c-Myc transcriptional activity by vIRF-3 has to be considered.

The protein encoded by clone C19, identified in yeast two-hybrid screening, is almost identical to MM-1α isoform. Of all MM-1 isoforms (α, β, γ, and δ) identified to date, MM-1α is the most predominant type, which, together with MM-1, exerts the highest degree of repression activity on c-Myc (39). Both MM-1 and MM-1α are nuclear proteins (39), and the fact that MM-1α, c-Myc, and vIRF-3 are all localized in the nuclei of PEL cells further strengthens our hypothesis that vIRF-3 is involved in stimulation of the transcriptional activity of c-Myc.

The c-Myc de-regulation frequently occurs in lymphomas due to chromosomal translocations and gene amplification. Nearly all cases of Burkitt lymphoma carry the c-myc translocated to the immunoglobulin loci (54). In addition, c-Myc transactivation is a common target of some tumor viruses (55, 56). Although c-Myc de-regulation appears to be critical for the development of lymphoma, there is no evidence of any c-myc locus rearrangements in KSHV-associated PEL (13, 57, 58). Therefore, the stimulatory effect of vIRF-3 on the c-Myc-mediated transcription may represent an important mechanism by which KSHV stimulates the c-Myc function and consequently promotes cell growth and transformation. Additional experiments are needed to determine whether the association of vIRF-3 with c-Myc can also alter the DNA-binding specificity of c-Myc, which may result in the activation of a distinct set of genes that are not regulated by c-Myc alone. Recently, two groups linked another KSHV-encoded latent gene, LANA, to impaired c-Myc degradation in PEL cells (59, 60). They showed that LANA stabilizes c-Myc protein by preventing its phosphorylation on threonine 58 and simultaneously stimulates its phosphorylation at serine 62, which leads to c-Myc transcriptional activation. Taken together, it appears that there are multiple mechanisms by which KSHV latent genes target the function of c-Myc, which may have a critical role in KSHV-associated lymphomagenesis.

In summary, our data clearly demonstrate that the KSHV-encoded vIRF-3 stimulates the transcriptional activity of the c-Myc oncogene most likely by two mechanisms. First, by sequestering the MM-1α protein, it releases c-Myc from MM-1α-mediated transcriptional repression. Second, as part of the transcription complex assembled on the cdk4- and E-box-containing promoters, it directly participates in the activation of c-Myc-mediated transcription. Due to the fact that we were unable to detect the association of MM-1α with the cdk4 promoter, it appears that these two activities reported for vIRF-3 represent independent events. Detailed mapping and comparison of the vIRF-3 binding sites to c-Myc and MM-1α followed by their mutational analyses may further clarify the mechanism by which vIRF-3 stimulates the function of c-Myc.

Most of the experimental evidence suggests that neoplastic transformation is a multistep process that requires inactivation of both retinoblastoma (pRb) and p53 tumor suppressors as well as the activation of one or more dominant oncogenes (61). In vitro studies of Rivas et al. (10) suggested that vIRF-3 (LANA2) interacts with p53 and inhibits p53-induced apoptosis. However, in vivo interaction between vIRF-3 and p53 in the cells remains to be demonstrated. The direct inhibition of pRb- and p53-signaling pathways by KSHV latent proteins, v-cyclin and LANA, may also contribute to the proliferation and neoplastic transformation of KSHV-infected B-cells (62–65). Furthermore, vFLIP constitutively activates NFκB and, therefore, may be essential for the survival of infected B cells (66). Thus, it seems likely that KSHV-encoded latent proteins contribute to the genesis of B-cell lymphoma both by down-modulating the function of tumor suppressors as well as up-regulating the transcriptional activity of the c-Myc oncogene. It remains to be determined whether a single KSHV protein can initiate or sustain the tumorigenic phenotype of these cells or whether the expression of several viral proteins is required for tumorigenicity.

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