

Kaposi's Sarcoma–Associated Herpesvirus Viral IFN Regulatory Factor 1 Inhibits Transforming Growth Factor- β Signaling

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Abstract

Kaposi's sarcoma-associated herpesvirus, also called human herpesvirus 8, has been implicated in the pathogenesis of Kaposi's sarcoma, body cavity-based primary effusion lymphoma, and some forms of multicentric Castleman's disease. The Kaposi's sarcoma-associated herpesvirus open reading frame K9 encodes viral IFN regulatory factor 1 (vIRF1), which functions as a repressor of IFN-mediated signal transduction. vIRF1 expression in NIH 3T3 cells leads to transformation and consequently induces malignant fibrosarcoma in nude mice, suggesting that vIRF1 is a strong oncoprotein. Here, we show that vIRF1 inhibited transforming growth factor- β (TGF- β) signaling via its targeting of Smad proteins. vIRF1 suppressed TGF- β -mediated transcription and growth arrest. vIRF1 directly interacted with both Smad3 and Smad4, resulting in inhibition of their transactivation activity. Studies using vIRF1 deletion mutants showed that the central region of vIRF1 was required for vIRF1 association with Smad3 and Smad4 and that this region was also important for inhibition of TGF- β signaling. In addition, we found that vIRF1 interfered with Smad3-Smad4 complex formation and inhibited Smad3/Smad4 complexes from binding to DNA. These results indicate that vIRF1 inhibits TGF- β signaling via interaction with Smads. In addition, the data indicate the TGF- β pathway is an important target for viral oncoproteins. (Cancer Res 2005; 65(5): 1738-47)

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), also called human herpesvirus 8, has been implicated in the development of Kaposi's sarcoma lesions, body cavity-based primary effusion lymphoma, and a subset of multicentric Castleman's disease (1–3). The KSHV genomic structure is similar to that of other γ herpesviruses. Interestingly, the KSHV genome has a unique series of nonstructural genes, which have been pirated from the host genome (4, 5). Viral infection induces a potent antiviral response mediated by IFNs, which play an important role in host immune surveillance. IFNs exhibit a wide range of biological activities, including cell growth inhibition and immune activation. Viruses have developed a variety of strategies to cope with the antiviral effects of IFNs (6). IFN signaling is regulated by IFN regulatory factors, which are a family of DNA binding proteins that act as activators or repressors (7). The KSHV genome contains at least

three open reading frame (ORF) encoding proteins with homology to IFN regulatory factor, including ORF K9–encoded viral IFN regulatory factor 1 (vIRF1), ORF K11.1–encoded vIRF2, and ORF K10.5–encoded vIRF3/latency-associated nuclear antigen 2 (8–12). vIRF1 protein comprises 449 amino acids with a NH₂-terminal region containing a conserved tryptophan-rich DNA binding region and displaying 70% identity to the IFN consensus sequence binding protein (4). Several groups have shown that vIRF1 functions as a negative regulator of cellular IFN-induced signaling (10, 13, 14). vIRF1 expression in NIH 3T3 cells leads to transformation and consequently induces malignant fibrosarcoma in nude mice, suggesting that vIRF1 is a potent oncoprotein (10, 14). In addition, vIRF1 associates with the tumor suppressor p53 protein, leading to the repression of p53-dependent transcription and apoptosis (15, 16). Furthermore, vIRF1 protein associates with p300/cAMP-responsive element binding protein, resulting in the inhibition of transactivation of cAMP-responsive element binding protein, histone acetyltransferase activity of p300, and formation of transcriptionally active IRF3-p300/cAMP-responsive element binding protein complexes (17–20). Recently, we showed that vIRF1 also interacts with a newly characterized cell death regulator, GRIM19, leading to inhibition of IFN/retinoic acid–induced cell death (21, 22). These reports collectively show that vIRF1 augments tumorigenicity.

Members of the transforming growth factor- β (TGF- β) family regulate a variety of biological processes, including cell growth, differentiation, matrix production, and apoptosis (23–25). TGF- β initiates signaling by assembling receptor serine/threonine kinases, termed type I and II receptors. The type I receptor activates members of the Smad family of tumor suppressors, termed receptor-regulated Smads, which include Smad2 and Smad3 in TGF- β signaling. The activated receptor-regulated Smads form complexes with a common mediator Smad, Smad4, and translocate to the nucleus, where they are involved in regulating transcription of target genes (23, 25, 26). TGF- β inhibits cell proliferation by regulating two classes of genes. Firstly, TGF- β -activated Smad complexes target the promoter of the *c-myc* gene, leading to transcriptional inhibition of *c-myc*. Secondly, activated Smad complexes are involved in induction of two cyclin-dependent kinase inhibitors, p15 and p21 (27–30). Smad proteins contain two conserved globular domains, the Mad homology 1 domain that binds DNA and the Mad homology 2 domain that binds the transcriptional coactivator p300/cAMP-responsive element binding protein in competition with corepressors TGIF, Ski, and SnoN (25).

Because TGF- β plays an important role in cell growth and differentiation, we investigated whether vIRF1 can modulate TGF- β signaling. In this study, we found that vIRF1 inhibited TGF- β -mediated transcription and growth arrest. In addition, vIRF1 physically associated with Smad3 and Smad4, thereby inhibiting the Smad3-Smad4 interaction. These findings reveal that KSHV vIRF1 functions as a negative regulator of the TGF- β pathway.

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Materials and Methods

Plasmids. The vIRF1 expression plasmid (pcDNA3-vIRF1) and the glutathione *S*-transferase (GST)-tagged vIRF1 expression plasmid (pEBG-vIRF1) were described previously (22). FLAG-vIRF1 and its mutants plasmids were generated by subcloning the corresponding sequences into the *EcoRI/XhoI* sites of pME18S. The pCGN2-HA-vIRF1 construct expressing hemagglutinin (HA)-tagged vIRF1 was generated by subcloning into the *XbaI/BamHI* sites of pCGN2-HA. The following plasmids were kind gifts from Dr. Joan Massague (Cancer Biology and Genetics Program, Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, NY): z p3TP-Lux, pCS2-FLAG-Smad3, pCMV5-HA-Smad4, and pCMV5-HA-TGF- β RI-[TD]. The pSBE4-Luc reporter construct was a gift from

Dr. Bert Vogelstein (Howard Hughes Medical Institute and Sidney Kimmel Cancer Center, Johns Hopkins Medical Institutions, Baltimore, MD). The pGEX-4T-1-Smad3 and pGEX-4T-1-Smad4 constructs were generated by inserting the appropriate sequences into the *EcoRI/XhoI* sites of pGEX-4T-1 (Amersham Pharmacia Biotech, Uppsala, Sweden). The pcDNA3-Smad3 and pcDNA3-Smad4 expression plasmids were generated by subcloning into the *EcoRI/XhoI* sites of pcDNA3. To generate GAL4-Smad3 and GAL4-Smad4 expression plasmids, the corresponding sequences were subcloned into the *EcoRI/NotI* sites of pCMV-G4. The GST-Smad3 and GST-Smad4 expression plasmids were constructed by inserting the required sequences into the *BamHI/NotI* sites of pEBG. The pFR-Luc and pRSV/ β -gal plasmids were described previously (22).

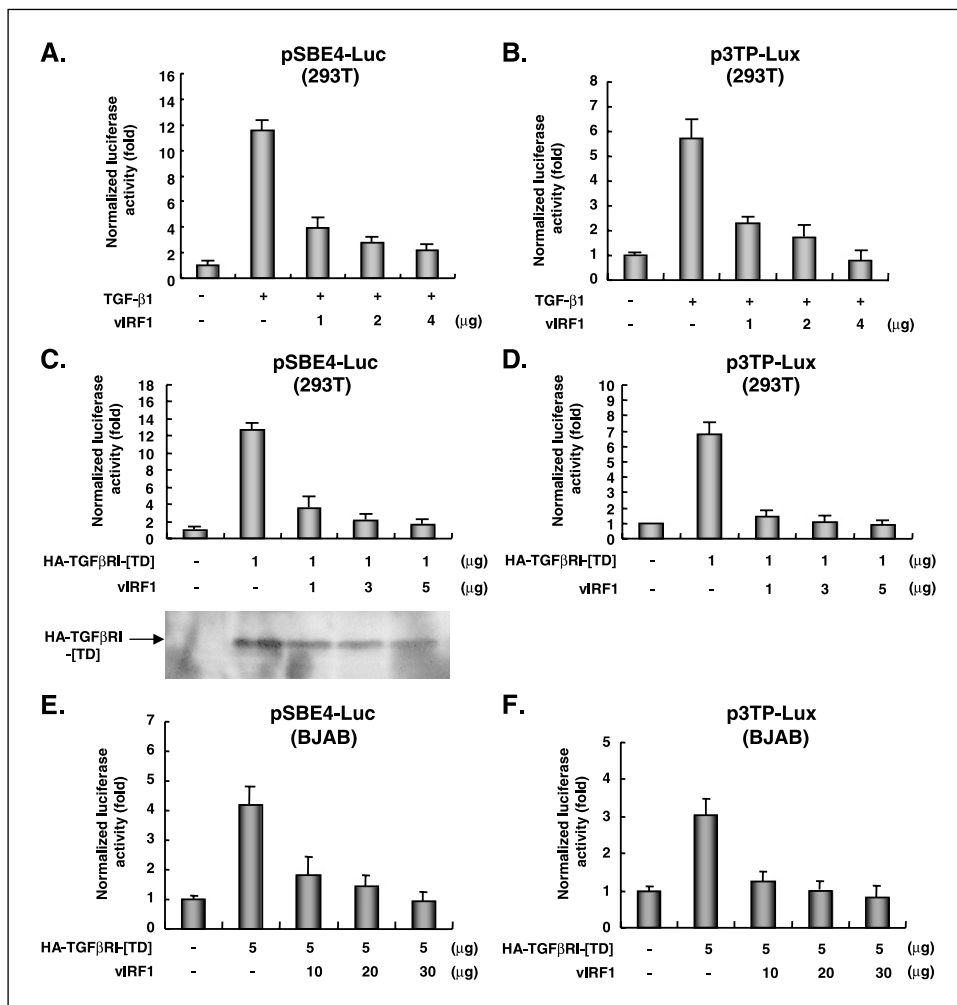


Figure 1. Repression of TGF- β -mediated transcription by vIRF1. **A**, TGF- β 1-stimulated expression of a synthetic reporter is inhibited by vIRF1. 293T cells in 35 mm dishes were cotransfected with 1 μ g pSBE4-Luc (a plasmid containing four Smad binding elements fused to a luciferase gene), 0.5 μ g pRSV/ β -gal (a Rous sarcoma virus β -galactosidase expression plasmid), and increasing amounts of pcDNA3-vIRF1 (an expression plasmid encoding vIRF1). After 24-hour transfection, cells were treated with or without 1 ng/mL TGF- β 1 for 24 hours and luciferase activity was measured. **B**, TGF- β 1-stimulated expression of p3TP-Luc containing the plasminogen activator inhibitor-1 promoter was inhibited by vIRF1. 293T cells in 35 mm dishes were cotransfected as indicated with 0.5 μ g p3TP-Luc and other plasmids listed in **A**. After 24-hour transfection, cells were treated with TGF- β 1 and assayed as described in **A**. **C**, pSBE4-Luc expression driven by the constitutively active TGF- β type I receptor was repressed by vIRF1. 293T cells in 60 mm dishes were cotransfected as indicated with pSBE4-Luc (1 μ g), pRSV/ β -gal (0.5 μ g), HA-TGF- β RI-[TD] (1 μ g), and increasing amounts of an expression plasmid encoding vIRF1. After 24-hour transfection, luciferase activity was measured. Equal amounts of total cell extracts were resolved by SDS-PAGE and subjected to HA-specific immunoblotting. **D**, p3TP-Luc expression driven by the constitutively active TGF- β type I receptor was repressed by vIRF1. 293T cells in 60 mm dishes were cotransfected as indicated with p3TP-Luc (1 μ g) and the plasmids listed in **C**. Assays were done as described in **C**. **E**, pSBE4-Luc expression driven by the constitutively active TGF- β type I receptor was repressed by vIRF1 in BJAB cells. BJAB cells were cotransfected as indicated with pSBE4-Luc (5 μ g), pcDNA3/ β -gal (5 μ g), HA-TGF- β RI-[TD] (5 μ g), and increasing amounts of an expression plasmid encoding vIRF1. Luciferase activity was measured at 24 hours after transfection. **F**, p3TP-Luc expression driven by the constitutively active TGF- β type I receptor was repressed by vIRF1 in BJAB cells. Assays were done as described in **E**, except that p3TP-Luc reporter was used. Luciferase activity was measured with a luminometer in all experiments. Total amount of transfected DNA in each experiment was kept constant by the addition of blank vector (pcDNA3). Activity of the reporter alone was set to a value of 1, and luciferase activity changes are presented as fold activation. Luciferase activity was normalized to β -galactosidase activity. Columns, mean of two (in BJAB cells) or three (in 293T cells) independent experiments; bars, SD.

Cell Culture, Transfection, and Reporter Assays. 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum. The TGF- β -sensitive cell line Mv1Lu (mink lung epithelial cell line, CCL-64, American Type Culture Collection, Manassas, VA) was grown in MEM containing 10% fetal bovine serum. BJAB and BCBL-1 cells were maintained in RPMI 1640 containing 10% fetal bovine serum. Transfections were done using either the calcium phosphate method (31) or LipofectAMINE Plus reagents (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. BJAB cells were transfected by electroporation as described previously (15). To generate cells stably expressing vIRF1, Mv1Lu cells were transfected with pcDNA3 (control) or pcDNA3-vIRF1 and were selected for 3 weeks in complete medium supplemented with 1 mg/mL G418 (Invitrogen). Polyclonal populations were grown and assayed for stable transgene expression. In reporter assays, the transfected plasmids were prepared using the midprep procedure (Qiagen, Hilden, Germany), and the total amount of plasmid was adjusted with a blank plasmid lacking the cDNA to be expressed. After 24-hour transfection, cells were treated with TGF- β 1 (R&D Systems, Minneapolis, MN). Equal amounts of cell extracts were employed for detection of luciferase activity. Each assay was normalized using β -galactosidase activity.

Growth Inhibition Assays. Mv1Lu cells in six-well plates were incubated for 24 hours in the absence or presence of TGF- β 1. During the last 3 hours, cells were labeled with 1 μ Ci/mL [3 H]thymidine (Amersham Pharmacia Biotech). 3 H-Labeled cells were washed twice with cold PBS, and the DNA was precipitated by incubating the cells in cold 10% trichloroacetic acid for 10 minutes. The trichloroacetic acid-precipitated DNA was washed twice with cold 10% trichloroacetic acid and incubated with rocking for 30 minutes in 500 μ L of 1% SDS, 0.1 N NaOH. The solution was added to 4.5 mL scintillation cocktail, and the precipitated 3 H-labeled DNA was quantified by scintillation counting.

In vivo Binding Assays. 293T cells were transiently cotransfected with either GST or GST-vIRF1 in combination with FLAG-Smad3 or HA-Smad4. After 48-hour transfection, cells were lysed with EBC buffer [50 mmol/L Tris-HCl (pH 7.5), 120 mmol/L NaCl, 0.5% NP40, 50 mmol/L NaF, 200 μ mol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride] and incubated with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) at 4°C for 2 hours with rocking. Bound protein complexes were washed thrice with EBC buffer and heated to 95°C for 5 minutes in SDS sample buffer. Western blots were done using anti-HA, anti-FLAG, and anti-GST mouse monoclonal antibodies. BJAB and 12-*O*-tetradecanoylphorbol-13-acetate-induced BCBL-1 cells were lysed with EBC buffer and immunoprecipitated with anti-Smad3, anti-Smad4, or anti-HA mouse monoclonal antibodies as described above. Samples were immunoblotted with anti-vIRF1 rabbit polyclonal antibody.

In vitro Binding Assays. Wild-type GST and GST fusion proteins were prepared by induction of *Escherichia coli* containing a fusion vector with 1 mmol/L isopropyl- β -D-galactopyranoside. After lysis by sonication, GST and GST-fusion proteins were bound to glutathione-Sepharose 4B beads, washed with PBS, and eluted with elution buffer [50 mmol/L Tris-HCl (pH 8.0), 25 mmol/L glutathione]. 35 S-labeled proteins were synthesized *in vitro* using the TNT-coupled transcription-translation system (Promega, Madison, WI) as described by the manufacturer. Glutathione-Sepharose 4B fusion protein was incubated with 35 S-labeled proteins in 500 μ L binding buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA (pH 8.0), 2.5 mmol/L DTT, 0.7 mg/mL bovine serum albumin, 0.1% NP40] at 4°C for 3 hours. The precipitated protein complexes were washed five times with binding buffer, SDS sample buffer was added, and the proteins were analyzed by SDS-PAGE followed by autoradiography.

Nuclear Extract Preparation. Nuclear extracts were prepared from 293T cells. Briefly, cells from 100 mm dishes were washed with cold PBS and scraped into test tubes. Cells were again washed and then suspended in 300 μ L cold HB cell lysis buffer [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl₂, 1.5 mmol/L DTT, 0.1 mmol/L phenylmethylsulfonyl fluoride, 1 μ g/mL leupeptin]. The suspended cells were incubated on ice for 15 minutes and then lysed using a Dounce all-glass homogenizer (30 strokes). After centrifugation, the pellets were washed with cold HB cell lysis buffer and

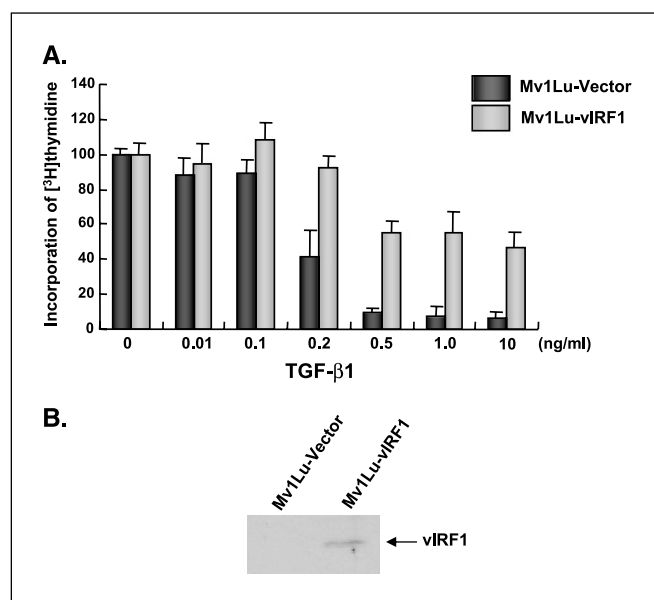


Figure 2. Suppression of TGF- β -mediated growth arrest by vIRF1. **A.** [3 H]thymidine incorporation as a function of various concentrations of TGF- β 1 (expressed as a percentage of the untreated control). vIRF1-expressing Mv1Lu cells and vector control-transfected Mv1Lu cells were treated with the indicated concentrations of TGF- β 1 for 21 hours, after which [3 H]thymidine was added and the cells were harvested 3 hours later for radioactive counting. **Columns,** mean of two independent experiments; **bars,** SD. **B.** Stable expression of vIRF1 in Mv1Lu cells. Total cell extracts were prepared from Mv1Lu cells expressing vector or vIRF1, resolved using SDS-PAGE, and immunoblotted using an anti-vIRF1 antibody.

resuspended in 100 μ L cold buffer C [30 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl₂, 0.3 mmol/L EDTA, 450 mmol/L NaCl, 10% glycerol, 0.1% NP40, 1 mmol/L DTT, 0.1 mmol/L phenylmethylsulfonyl fluoride, 1 μ g/mL leupeptin]. Nuclear membranes were lysed using 15 strokes of Dounce all-glass homogenizer. After centrifugation, the supernatant was collected, added to an equal volume of buffer D [30 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl₂, 0.3 mmol/L EDTA, 10% glycerol, 1 mmol/L DTT, 0.1 mmol/L phenylmethylsulfonyl fluoride, 1 μ g/mL leupeptin], and frozen at -70°C until ready for use.

Electrophoretic Mobility Shift Assays. The following wild-type double-stranded CAGA oligonucleotide sequence and its complementary strand were used as probes: 5'-TCGAGAGCCAGACAAGGAGCCAGACAAGGAGC-CAGACAC-3'. The following mutant double-stranded CAGA oligonucleotide sequence and its complementary strand were used as probes: 5'-TCGAGAGCTACATAAGGAGCTACATAAGGAGCTACATAC-3'. Each oligonucleotide was end labeled with [γ - 32 P]ATP using T4 kinase (Promega). Binding reactions were done at room temperature for 30 minutes in a total volume of 25 μ L reaction mixture containing 20 mmol/L HEPES (pH 7.9), 30 mmol/L KCl, 4 mmol/L MgCl₂, 0.1 mmol/L EDTA, 10% glycerol, 4 mmol/L spermidine, 1 μ g poly(deoxyinosine-deoxycytosine), the labeled probe (50,000 cpm), and nuclear extracts. For competitive binding reactions, a 50-fold molar excess of unlabeled wild-type CAGA or mutant CAGA double-stranded oligonucleotide was preincubated in the reaction mixture at room temperature for 10 minutes before adding labeled probe. Analysis of binding complexes was done by electrophoresis in 5% native polyacrylamide gels with 0.5 \times Tris-borate electrophoresis buffer, and bands were visualized using autoradiography.

Results

vIRF1 Represses TGF- β -Mediated Transcription. Using cotransfection assays, we investigated whether vIRF1 modulated TGF- β -mediated transcription. 293T cells were transiently cotransfected with a vIRF1 expression plasmid plus either a pSBE4-Luc

reporter containing four Smad binding element sites or a p3TP-Lux reporter containing the plasminogen activator inhibitor-1 promoter. After 24-hour transfection, cells were treated with TGF- β 1 for 24 hours to stimulate TGF- β activity, after which cells were harvested and assayed for luciferase activity as a measure of TGF- β -mediated gene expression. In cells transfected with only the TGF- β -responsive reporters pSBE4-Luc or p3TP-Lux, we found that TGF- β 1 markedly increased luciferase activity, as expected (Fig. 1A and B). For both reporters, this TGF- β -stimulated luciferase activity was significantly inhibited in a dose-dependent manner following cotransfection of the vIRF1 expression plasmid (Fig. 1A and B). To further investigate this phenomenon, we stimulated TGF- β -induced transcription by engineering cells to express a HA-tagged constitutively active TGF- β type I receptor (HA-TGF- β RI-[TD]). Similar to the above data using exogenously added TGF- β 1, we found that expression of HA-TGF- β RI-[TD] increased luciferase activity from pSBE4-Luc and p3TP-Lux reporter constructs and that these responses were inhibited in a dose-dependent manner by cotransfection of vIRF1 (Fig. 1C and D). The expression of HA-TGF- β RI-[TD] was

monitored by Western blot assays, which showed that the level of HA-TGF- β RI-[TD] was not altered by expression of vIRF1. The transcriptional repression was specific because vIRF1 did not affect GAL4-SP1-driven transcription (15). Transfection experiment was also done in BJAB B-cell lymphoma cell line. As in 293T cells, HA-TGF- β RI-[TD] increased luciferase activities from pSBE4-Luc and p3TP-Lux, and the activities were decreased by coexpression of vIRF1 (Fig. 1E and F). These results indicate that vIRF1 specifically represses TGF- β -mediated transcription.

vIRF1 Suppresses TGF- β -Mediated Growth Inhibition. TGF- β causes G₁ cell cycle arrest (23, 25, 32, 33). We investigated whether vIRF1 affected TGF- β antiproliferative activity. Mv1Lu cells were stably transfected with a blank vector (pcDNA3; control) or a vIRF1 expression plasmid (pcDNA3-vIRF1). Successfully transfected cells were selected by 3-week culturing in medium containing G418 (1 mg/mL). The effect of TGF- β on cell proliferation was determined using [³H]thymidine incorporation assays. We found that 0.2 ng/mL TGF- β 1 inhibited DNA synthesis in control cultures, whereas cells expressing vIRF1 were resistant to this TGF- β 1-induced growth inhibition

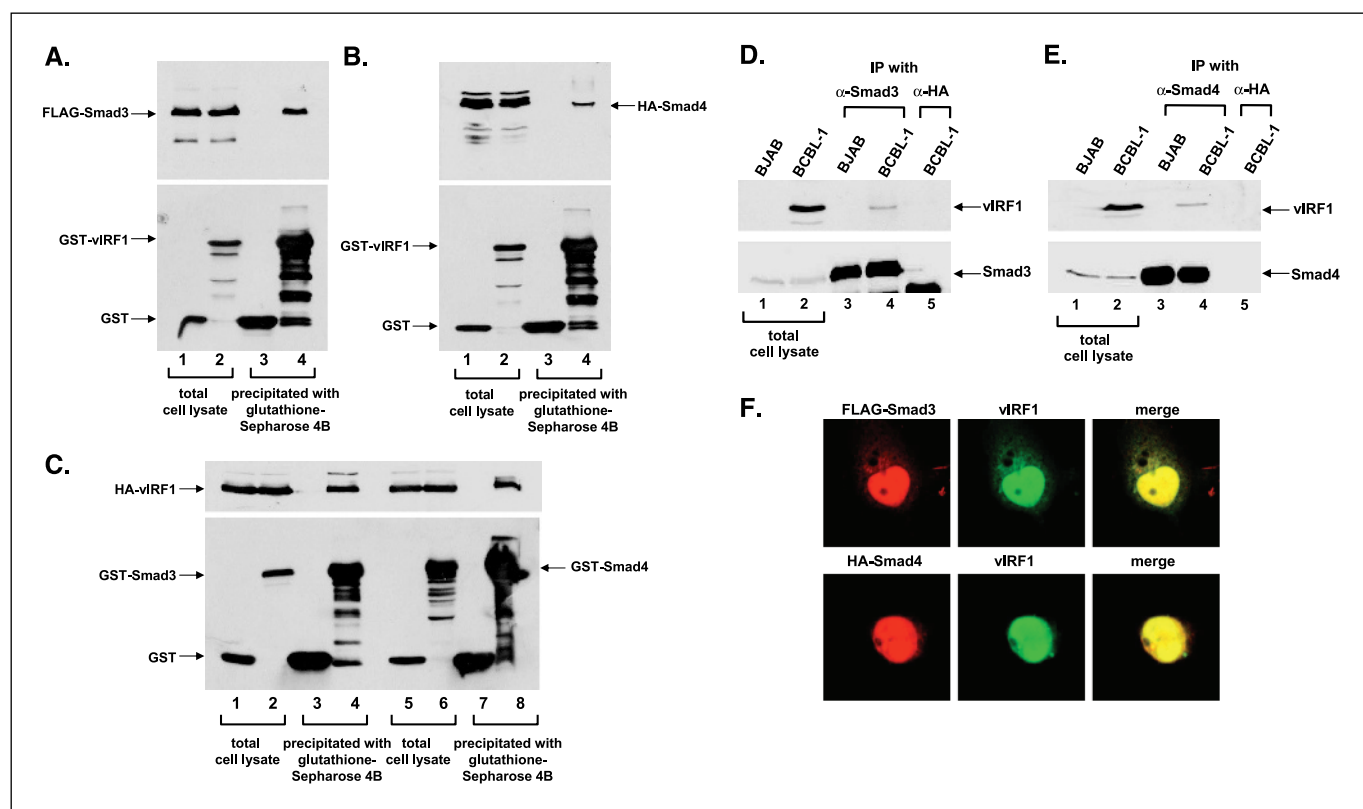


Figure 3. *In vivo* association of vIRF1 with Smad3 and Smad4. **A**, 293T cells were cotransfected with a FLAG-Smad3 expression plasmid in combination with either a GST expression plasmid or a GST-vIRF1 expression plasmid. Cell extracts were precipitated using glutathione-Sepharose 4B and the precipitates were washed and resolved by SDS-PAGE. GST fusion protein and FLAG-Smad3 were detected by Western blot using anti-FLAG (*top*) and anti-GST (*bottom*) antibodies, respectively. *Lanes 1 and 3*, GST with FLAG-Smad3; *lanes 2 and 4*, GST-vIRF1 with FLAG-Smad3. **B**, 293T cells were cotransfected with a HA-Smad4 expression plasmid in combination with either a GST expression plasmid or a GST-vIRF1 expression plasmid. Samples were prepared as described above. GST fusion protein and HA-Smad4 were immunoblotted using anti-HA (*top*) and anti-GST (*bottom*) antibodies, respectively. *Lanes 1 and 3*, GST with HA-Smad4; *lanes 2 and 4*, GST-vIRF1 with HA-Smad4. **C**, GST expression plasmid, GST-Smad3 expression plasmid, or GST-Smad4 expression plasmid was cotransfected with a HA-vIRF1 expression plasmid into 293T cells. Samples were prepared as described above. GST fusion proteins and HA-vIRF1 were immunoblotted using anti-HA (*top*) and anti-GST (*bottom*) antibodies, respectively. *Lanes 1 and 3*, GST with HA-vIRF1; *lanes 2 and 4*, GST-Smad3 with HA-vIRF1; *lanes 5 and 7*, GST with HA-vIRF1; *lanes 6 and 8*, GST-Smad4 with HA-vIRF1. **D**, BJAB and BCBL-1 cells (2×10^7) were stimulated for 48 hours with 12-*O*-tetradecanoylphorbol-13-acetate and lysates were prepared. Total cell extracts were immunoblotted using anti-vIRF1 and anti-Smad3 antibodies (*lanes 1 and 2*, respectively). Cell extracts were immunoprecipitated using anti-Smad3 (*lanes 3 and 4*) and anti-HA (*lane 5*) monoclonal antibodies. vIRF1 was detected by Western blotting using an anti-vIRF1 antibody (*top*). **E**, same study as described in **D**, except that an anti-Smad4 antibody was used. **F**, colocalization of vIRF1 and Smads. 293T cells were cotransfected with expression plasmids containing FLAG-Smad3 and vIRF1. Cells were fixed, incubated with anti-FLAG and anti-vIRF1 antibody, and detected with TRITC-conjugated anti-mouse antibody and FITC-conjugated anti-rabbit antibody (*top*). Same experiment, except that HA-Smad4 and anti-HA antibody was used (*bottom*).

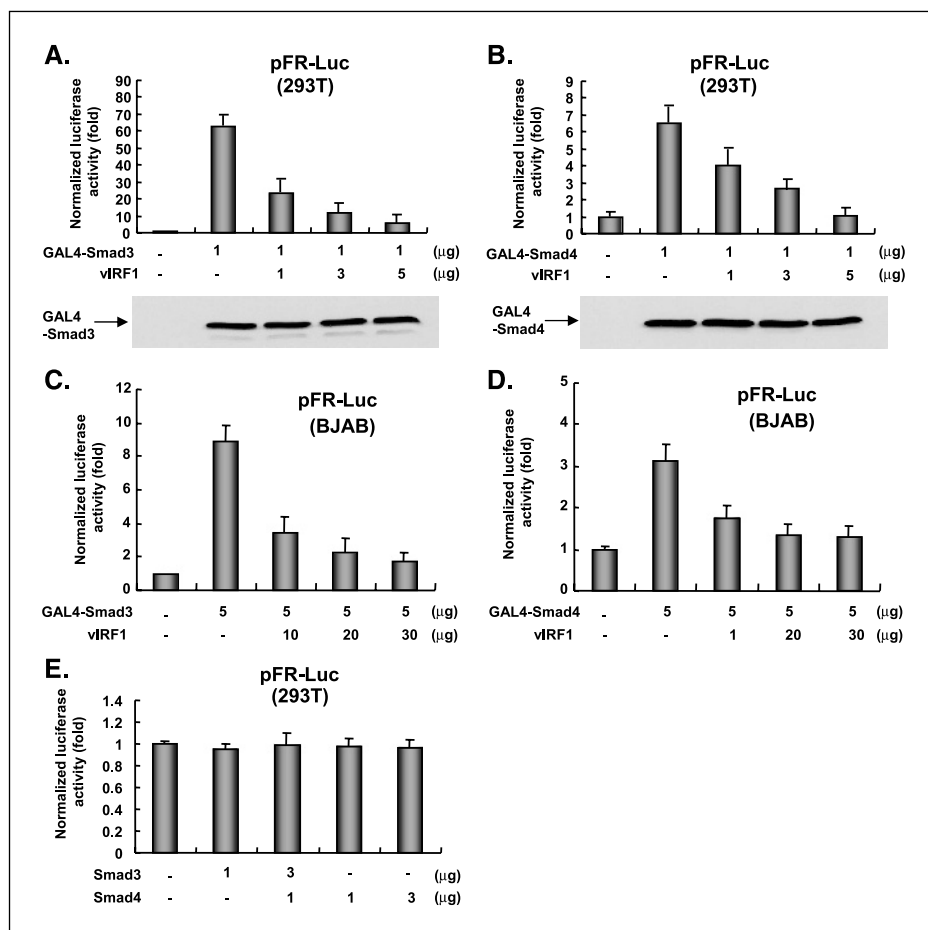


Figure 4. vIRF1 inhibits transcriptional activation by Smad3 and Smad4. *A*, 293T cells in 60 mm dishes were cotransfected with a GAL4-Luc reporter plasmid (pFR-Luc; 1 μ g), a GAL4-Smad3 expression plasmid (1 μ g), pRSV/ β -gal (0.5 μ g), and increasing amounts of an expression plasmid encoding vIRF1. After 24-hour transfection, cells were harvested and luciferase activity was measured. *B*, 293T cells were cotransfected with a GAL4-Luc reporter plasmid (pFR-Luc; 1 μ g), a GAL4-Smad4 expression plasmid (1 μ g), pRSV/ β -gal (0.5 μ g), and increasing amounts of an expression plasmid encoding vIRF1. After 24-hour transfection, cells were harvested and luciferase activity was measured. Total cell extracts were prepared from transfected cells, resolved by SDS-PAGE, and immunoblotted using an anti-GAL4 antibody. *C*, BJAB cells were cotransfected with pFR-Luc (10 μ g), a GAL4-Smad3 expression plasmid (5 μ g), pCDNA3/ β -gal (3 μ g), and increasing amounts of an expression plasmid encoding vIRF1. After 24-hour transfection, luciferase assays were done. *D*, same experiment as described in *C*, except that p3TP-Luc reporter was used. *E*, 293T cells were cotransfected with pFR-Luc (1 μ g) and increasing amounts of GAL4-fused Smad3 and GAL4-fused Smad4 expression plasmids. Luciferase assays were done as described above.

(Fig. 2A). vIRF1 expression was confirmed by Western blot assays using an anti-vIRF1 rabbit polyclonal antibody (Fig. 2B). These data suggest that vIRF1 overrides TGF- β -mediated growth inhibition.

vIRF1 Associates with Smad3 and Smad4. Because TGF- β signaling is primarily mediated by Smad proteins, we investigated whether vIRF1 directly acted on Smads to inhibit TGF- β signaling. 293T cells were cotransfected with GST or GST-vIRF1 expression plasmids plus either FLAG-Smad3 or HA-Smad4 expression plasmids. After 48-hour transfection, cell lysates were prepared and incubated with glutathione-Sepharose 4B beads to precipitate GST and GST-vIRF1, and the precipitated proteins were immunoblotted using anti-FLAG or anti-HA antibodies. We found that FLAG-Smad3 and HA-Smad4 proteins coprecipitated with GST-vIRF1 but not with GST alone (Fig. 3A and B, top). Expression of GST, GST-vIRF1, FLAG-Smad3, and HA-Smad4 was monitored using Western blot assays (Fig. 3A and B). In a reciprocal experiment, 293T cells were cotransfected with GST, GST-Smad3, or GST-Smad4 expression plasmids plus HA-vIRF1 expression plasmids. Cells were lysed, incubated with glutathione-Sepharose 4B, and immunoblotted with anti-HA antibody. HA-vIRF1 coprecipitated with GST-Smad3 and GST-Smad4 but not with GST (Fig. 3C, top). To examine whether vIRF1 and Smad proteins interact under conditions not involving transient enforced expression, we did coimmunoprecipitation assays in KSHV-infected BCBL-1 cells. Because vIRF1 is a lytic protein (34, 35), we induced vIRF1 expression by 12-*O*-tetradecanoylphorbol-13-

acetate stimulation. BJAB and 12-*O*-tetradecanoylphorbol-13-acetate-stimulated BCBL-1 cells were lysed and immunoprecipitated with anti-Smad3, anti-Smad4, and anti-HA (control) antibody. The precipitated proteins were immunoblotted with anti-vIRF1 antibody. We found that vIRF1 coimmunoprecipitated with both Smad3 and Smad4 in KSHV-infected BCBL-1 cells (Fig. 3D and E, top, lane 4). In contrast, vIRF1 was not detected in KSHV-negative BJAB cell extracts or following immunoprecipitation with anti-HA antibodies (Fig. 3D and E, top, lanes 3 and 5). Expression of Smad3 and Smad4 in both BJAB and BCBL-1 cells was confirmed by Western blot assay using anti-Smad3 and anti-Smad4 antibodies (Fig. 3D and E, bottom). To determine whether vIRF1 and Smads protein complexes colocalize, we examined their subcellular localization by immunofluorescence confocal microscopy. 293T cells were cotransfected with vIRF1 expression plasmid together with either FLAG-Smad3 or HA-Smad4 expression plasmids. As shown in Fig. 3F, vIRF1 was colocalized with both FLAG-Smad3 and HA-Smad4. These results show that vIRF1 associates with both Smad3 and Smad4 *in vivo*.

vIRF1 Represses Transactivation Activity of Smads. Because vIRF1 seemed to directly associate with Smad3 and Smad4, we investigated whether vIRF1 modulated the transcriptional activation activities of Smad3 and Smad4. We did transient cotransfection assays in 293T cells using a GAL4-Smad3 expression plasmid and the reporter plasmid pFR-Luc, which contains five GAL4 binding sites. Coexpression of these plasmids showed that GAL4-fused Smad3 activated luciferase activity >60-fold that of

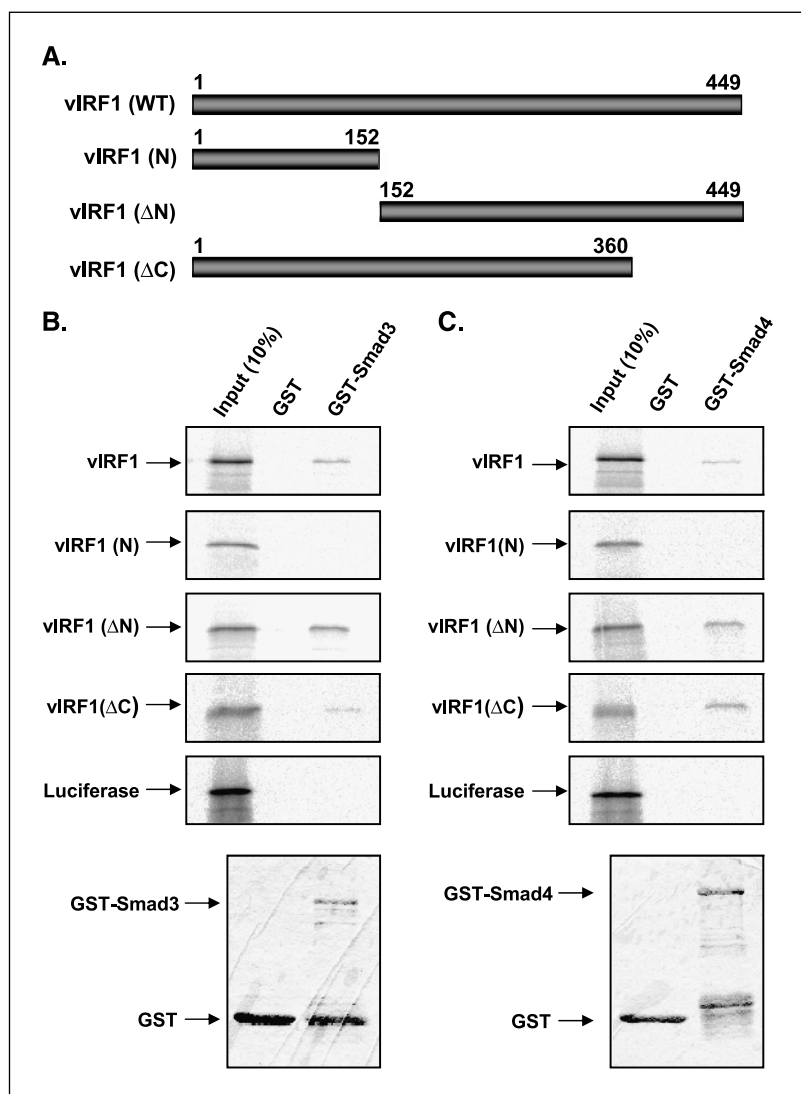


Figure 5. The central region of vIRF1 is required for vIRF1 interactions with Smad3/Smad4. *A*, schematic representation of vIRF1 and its deletion mutants. *B*, GST pull-down assays were done using GST-Smad3, ³⁵S-labeled *in vitro*-translated vIRF1, vIRF1 mutants, and luciferase (control). Input (10%) and GST pull-down mixtures were resolved by SDS-PAGE and visualized using autoradiography. *C*, same studies as described in *B*, except that GST-Smad4 was used. GST fusion proteins used in GST pull-down assays were visualized using Coomassie blue staining (*B* and *C*, bottom).

control (Fig. 4A). These data indicate Smad3 functions as a strong transactivator when tethered to a promoter. We found that cotransfection of a vIRF1 expression plasmid repressed the GAL4-Smad3-driven luciferase activity in a dose-dependent manner (Fig. 4A). We did similar cotransfection reporter assays in 293T cells using a GAL4-Smad4 expression plasmid. Again, we found that GAL4-Smad4 increased luciferase activity and that this activation was dose-dependently repressed by cotransfection of the vIRF1 expression plasmid (Fig. 4B). GAL4-Smad3 and GAL4-Smad4 expression was monitored using Western blot assays, which revealed that the levels of the GAL4 fusion proteins remained unchanged in the presence of vIRF1 (Fig. 4A and B). Transfection experiments were also done in BJAB cells. As in 293T cells, vIRF1 repressed luciferase activity driven by GAL4-Smad3 and GAL4-Smad4 (Fig. 4C and D). In control experiment, GAL4-fused Smad3 and GAL4-fused Smad4 did not affect the luciferase activity from pFR-Luc (Fig. 4F). These data suggest that vIRF1 inhibits the transcriptional activation activity of Smad proteins.

The Central Region of vIRF1 Is Necessary for vIRF1-Smads Interactions. To determine the region of vIRF1 that is required for interaction with Smad3 and Smad4, we constructed a series

of vIRF1 deletion mutants; vIRF1-N (amino acids 1-152), vIRF1- Δ N (amino acids 152-449), and vIRF1- Δ C (amino acids 1-360; Fig. 5A). GST pull-down assays were done using ³⁵S-labeled *in vitro*-translated vIRF1 and its mutants. We measured binding of *in vitro*-translated vIRF1, vIRF1 mutants, and luciferase (negative control) to GST-Smad3 immobilized on glutathione-Sepharose 4B beads. We found that GST-Smad3 interacted with wild-type vIRF1, vIRF1- Δ N, and vIRF1- Δ C but not with vIRF1-N or luciferase (Fig. 5B). Similarly, immobilized GST-Smad4 was found to bind to vIRF1, vIRF1- Δ C, and vIRF1- Δ N (Fig. 5C). GST and GST fusion proteins were visualized using SDS-PAGE followed by Coomassie blue staining (Fig. 5B and C). These data indicate that the central region of vIRF1 is required for vIRF1-Smads interactions.

vIRF1-Smads Interactions Are Important for Inhibition of TGF- β Signaling. We next investigated the importance of vIRF1-Smads interactions in suppressing TGF- β -mediated transcription. 293T cells were transiently cotransfected with combinations of a TGF- β -responsive reporter (p3TP-Lux), a constitutively active TGF- β type I receptor expression plasmid (HA-TGF- β RI-[TD]), vIRF1, and vIRF1 deletion mutants. We found that HA-TGF- β RI-[TD] expression induced luciferase transcription from the reporter

plasmid and that this induction was markedly repressed by cotransfection of wild-type ν IRF1, ν IRF1- Δ N, and ν IRF1- Δ C (Fig. 6A). In contrast, expression of ν IRF1-N did not affect luciferase reporter gene transcription (Fig. 6A). We showed that Smads can associate with wild-type ν IRF1, ν IRF1- Δ N, and ν IRF1- Δ C but not ν IRF1-N (Fig. 4A), indicating there is a correlation between Smads- ν IRF1 interactions and inhibition of TGF- β activity by ν IRF1. Expression of ν IRF1 and its deletion mutants was confirmed by Western blot assay (Fig. 6B). Our data suggest that ν IRF1-Smads interactions are important for repression of TGF- β -mediated transcription.

ν IRF1 Inhibits DNA Binding by the Smad3/Smad4 Complex.

We showed that ν IRF1 repressed TGF- β -mediated transcription (Fig. 1) and that ν IRF1 is directly associated with Smad3 and Smad4 (Fig. 3). We hypothesized that ν IRF1 inhibits TGF- β -mediated signaling by directly interacting with the Smad3/Smad4 complex to inhibit its association with DNA. Therefore, we investigated whether ν IRF1 could inhibit the Smad3/Smad4 complex from binding DNA. Previous work reported that the CAGA box within plasminogen

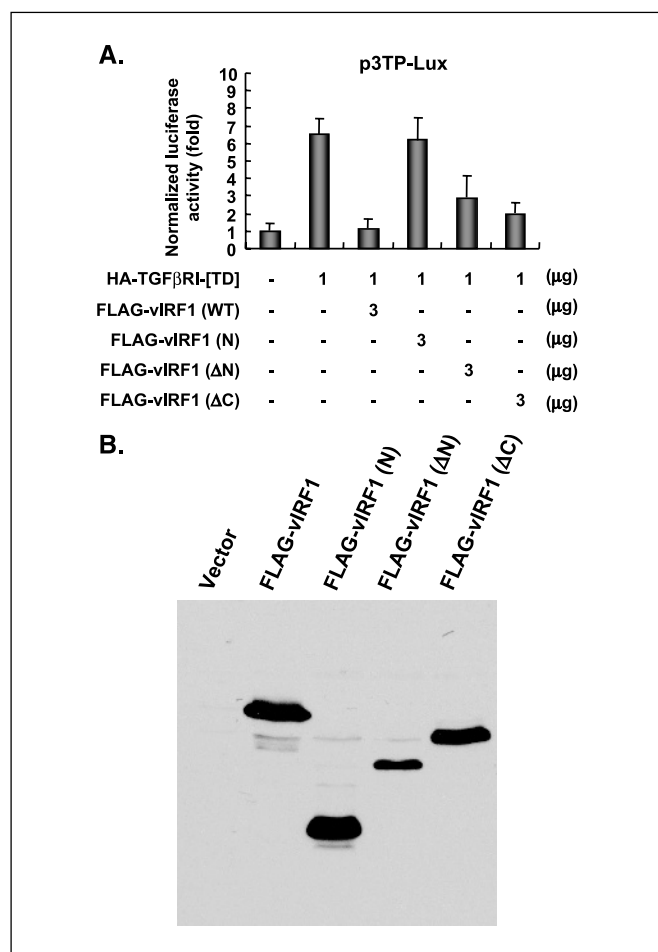


Figure 6. Contribution of ν IRF1 domains to the repression of TGF- β signaling. **A**, 293T cells in 60 mm dishes were cotransfected with p3TP-Lux (1 μ g), HA-TGF β RI-[TD] (1 μ g), pRSV/ β -gal (0.5 μ g), FLAG-tagged ν IRF1 expression plasmid, and its deletion mutant expression plasmids. After 24-hour transfection, cells were harvested and luciferase activity was measured using a luminometer. Luciferase activity was normalized to β -galactosidase activity. **Columns**, mean of three independent experiments; **bars**, SD. **B**, expression of FLAG-tagged ν IRF1 and its deletion mutants. Total cell extracts from transfected cells were resolved by SDS-PAGE and immunoblotted using an anti-FLAG antibody.

activator inhibitor-1 promoter is a TGF- β -inducible DNA element and that the Smad3/Smad4 complex directly binds to this CAGA box (36). We did electrophoretic mobility shift assays (EMSA) using transfected 293T cell nuclear extracts and CAGA sequences from the plasminogen activator inhibitor-1 promoter as probes (Fig. 7A). 293T cells were transfected to express combinations of FLAG-Smad3, HA-Smad4, ν IRF1, and HA-tagged constitutively active TGF- β type I receptor, and nuclear extracts were then prepared for EMSA. As expected, expression of constitutively active type I receptor along with Smad3/Smad4 markedly induced Smad3/Smad4-DNA binding (Fig. 7B, lane 2). Coexpression of ν IRF1 decreased Smad3/Smad4-DNA binding in a dose-dependent manner, suggesting that ν IRF1 interferes with this binding (Fig. 7B, lanes 3-5). To confirm that DNA-protein complexes contained the Smad3/Smad4 complex, we did supershift assays using an anti-FLAG antibody. Incubation with the anti-FLAG antibody resulted in a supershifted band, indicating that the shifted band contained FLAG-Smad3 protein (Fig. 7B, lane 6). To show that the DNA-protein complexes were specific for the CAGA sequence, we did EMSA using a CAGA mutant oligonucleotide as the probe. We found that the CAGA mutant oligonucleotide did not form a complex with Smad3/Smad4 protein (Fig. 7B, lane 8). We also did competition binding assays using competitor DNA. Preincubation with a 50-fold molar excess of unlabeled wild-type CAGA oligonucleotide diminished the amount of DNA-protein complex, whereas excess CAGA mutant oligonucleotide did not affect complex formation (Fig. 7B, lanes 9-11). The expression of transfected proteins was monitored using Western blot assays (Fig. 7C). To confirm that the shifted band was a complex of DNA and Smad3/Smad4, an additional supershift assay was done. Incubation with anti-HA antibody supershifted the specific band, suggesting that the shifted band contained HA-Smad4 protein (Fig. 7D). Collectively, the data indicate that ν IRF1 inhibits TGF- β -mediated Smad3/Smad4-DNA formation.

ν IRF1 Interferes with Smad3 and Smad4 Complex Formation. Because we found that ν IRF1 directly associates with Smad3 and Smad4, we investigated whether ν IRF1 could affect the complex formation between Smad3 and Smad4 during TGF- β stimulation. 293T cells were cotransfected with FLAG-Smad3, GST-Smad4, and HA- ν IRF1 expression plasmids. After 24-hour transfection, cells were stimulated TGF- β 1, and lysates were prepared using EBC buffer. Lysates were incubated with glutathione-Sepharose 4B beads to precipitate GST-Smad4, and the precipitated proteins were analyzed using Western blot assays. We found that TGF- β 1 induced Smad3 and Smad4 complex formation and that coexpression of ν IRF1 inhibited this event (Fig. 8, top, lanes 3 and 5). FLAG-Smad3, GST-Smad4, and HA- ν IRF1 expression was monitored by Western blot assays using anti-FLAG, anti-GST, and anti-HA monoclonal antibodies. These results suggest that ν IRF1 interferes with the complex formation between Smad3 and Smad4.

Discussion

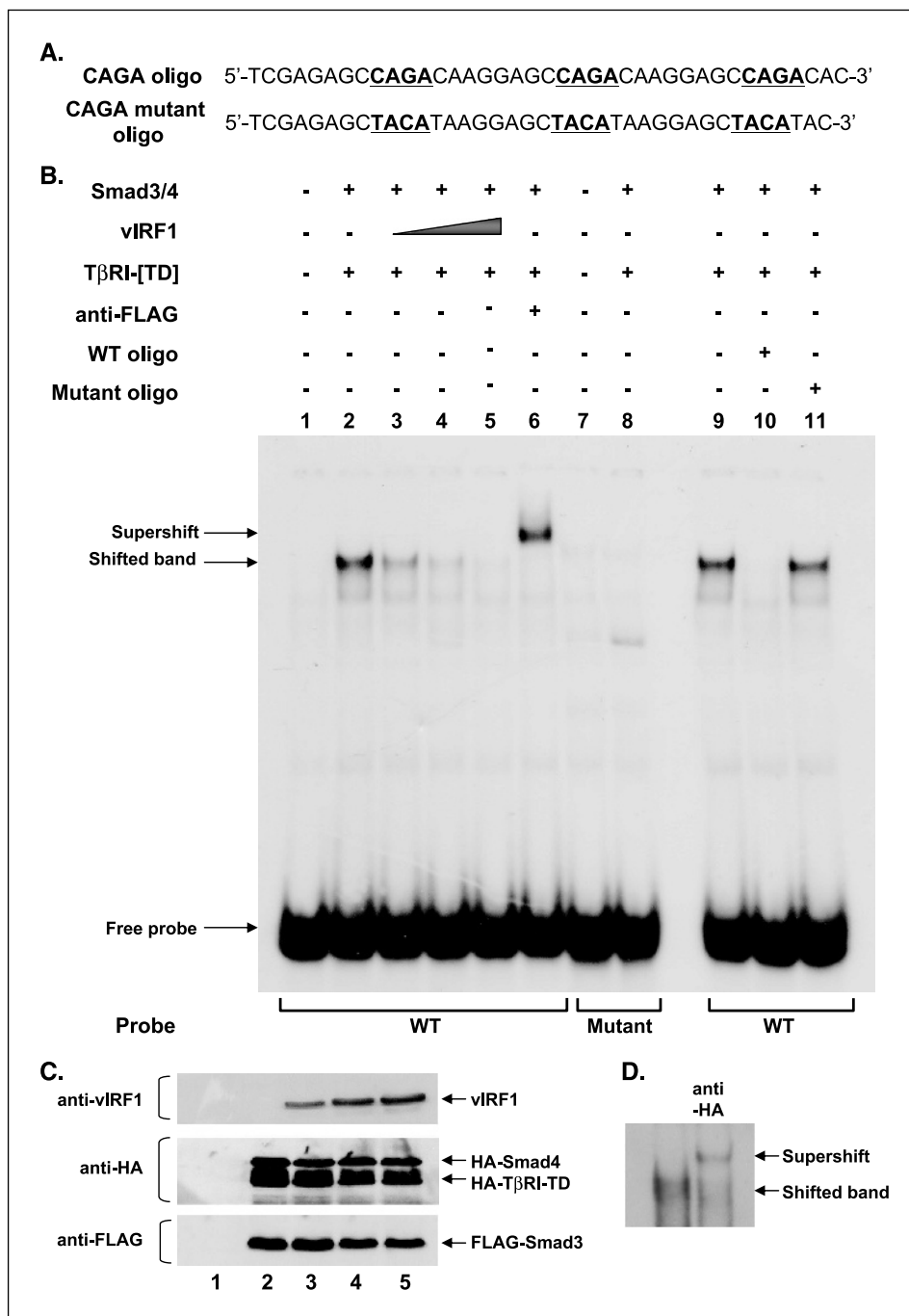
KSHV is implicated as an etiologic agent for a series of neoplastic disorders, including Kaposi's sarcoma, body cavity-based primary effusion lymphoma, and multicentric Castleman's disease, suggesting that it is a model of human DNA tumor viruses (1-3). It has been proposed that some KSHV viral proteins might contribute to tumor development and inhibition of programmed cell death. An intriguing feature of these viral

proteins is that most show significant homology to cellular proteins, suggesting that they were originally pirated from the host cells. These viral proteins include vBCL-2 (ORF 16), vIRF1 (ORF K9), vFLIP (ORF 71), vCyclin (ORF 72), vGPCR (ORF 74), kaposin (ORF K12), and K1 proteins (37, 38). At least four KSHV viral proteins transform cells in culture, including vIRF1, vGPCR, kaposin, and K1. Among these proteins, vIRF1 was suggested as being a strong candidate viral oncoprotein. Expression of vIRF1 induces cellular transformation in NIH 3T3 cells, resulting in morphologic change, loss of contact inhibition, colony formation in soft agar, and tumor induction in nude mice (10, 14). In addition, vIRF1 interacts with p53 and

GRIM19, resulting in inhibition of p53-dependent apoptosis and IFN/retinoic acid-induced cell death (15, 16, 22). These data partly provide a clue regarding the possible molecular mechanisms underlying vIRF1-induced transformation. Here, we showed that vIRF1 inhibited another tumor suppressor pathway, the TGF-β-stimulated signaling cascade. We found that vIRF1 suppressed TGF-β-mediated transcription and growth arrest. In addition, vIRF1 physically associated with Smad3 and Smad4, resulting in inhibition of the formation of Smad3/Smad4-DNA complexes and suppression of TGF-β-mediated signaling.

Inactivation of the TGF-β signaling pathway is important in the genesis of human malignances (25, 32, 33). During tumor

Figure 7. vIRF1 inhibits the Smad3/Smad4 complex from binding to DNA. *A*, probe sequences used in EMSAs. *B*, 293T cells were cotransfected with HA-TGF-βRI-[TD], FLAG-Smad3 and HA-Smad4 expression plasmids, and increasing amounts of a vIRF1 expression plasmid. After 24-hour transfection, nuclear extracts were prepared and equal amounts were used in EMSAs using ³²P-labeled CAGA oligonucleotide or CAGA mutant oligonucleotide probes. Shifted and supershifted bands labeled with anti-FLAG antibody. Competition assays were done by preincubation with excess (50-fold) unlabeled wild-type (WT) and mutant CAGA oligonucleotides (lanes 9-11). *C*, nuclear extracts used in EMSAs were analyzed using immunoblotting with anti-vIRF1, anti-HA, and anti-FLAG antibodies. *D*, shifted and supershifted bands labeled with anti-HA antibody.



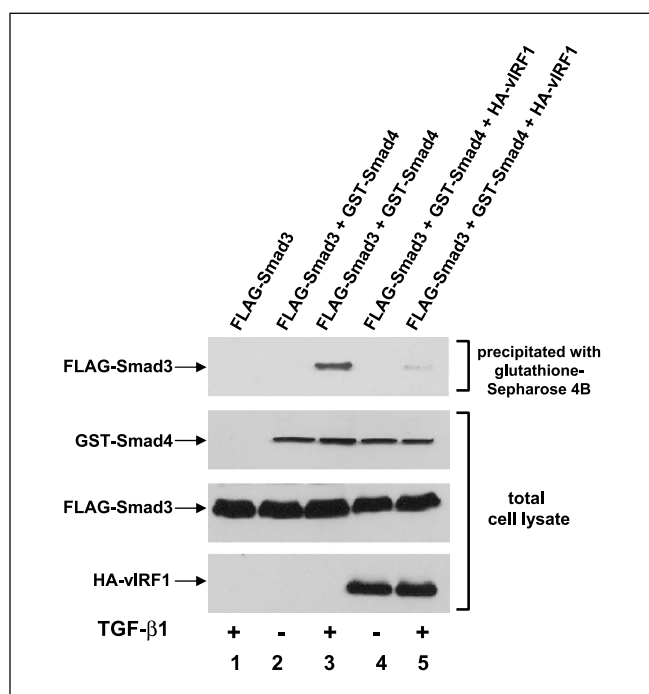


Figure 8. vIRF1 blocks complex formation between Smad3 and Smad4. 293T cells were transfected to express FLAG-tagged Smad3 and GST-Smad4 either without (lanes 2 and 3) or with (lanes 4 and 5) HA-vIRF1. After 24-hour transfection, where indicated, cells were treated with TGF- β 1 (5 ng/mL) for 2 hours. Cell extracts were prepared and precipitated using glutathione-Sepharose 4B beads, and precipitated proteins were analyzed using immunoblotting with an anti-FLAG antibody (top). Total expression of Smads and vIRF1 was monitored by Western blot assays using anti-GST, anti-FLAG, and anti-HA antibodies.

progression, many cancer cells tend to acquire a resistance to TGF- β -induced growth inhibition. It has been reported that nearly all pancreatic cancers and colon cancers have mutations in a component of the TGF- β signaling pathway (39–41). *In vivo* experiments with mice support the importance of TGF- β in tumor suppression. Mice carrying a heterozygous deletion in the *TGF- β* gene show a subtly altered proliferative phenotype with increased cell turnover in the liver and lung, and treatment of these mice with chemical carcinogens resulted in enhanced tumorigenesis (42). It has been also reported that transgenic mice overexpressing a dominant-negative mutant TGF- β type II receptor show enhanced tumorigenesis in the mammary gland and lung in response to carcinogen challenge (43). The pivotal role of TGF- β in tumor suppression would explain the oncogenic effects of vIRF1 targeting the TGF- β pathway.

Because TGF- β plays a central role in cell growth, differentiation, and immune cell regulation, it is a suitable target for several viral proteins that interfere with signal transduction and transcription control in infected cells. EBV-transformed human B cells are resistant to the growth inhibitory effects of TGF- β , and this

resistance is partly associated with down-regulation of the TGF- β receptor (44, 45). In addition, the presence of EBV latent membrane protein 1 results in loss of TGF- β -mediated transcription and growth inhibition (46, 47). Human T-cell lymphotropic virus type 1-infected human T cells are refractory to growth suppression by TGF- β (48). Interestingly, human T-cell lymphotropic virus type 1 Tax protein inhibits TGF- β signaling through association with Smad proteins (49). Human papillomavirus E7 protein also suppresses TGF- β signaling by blocking formation of Smad-DNA complexes through direct binding to Smad proteins (50). Here, we found that vIRF1 also targets Smad proteins to regulate TGF- β signaling. That Smad proteins are an important target for viral proteins, which deregulate the TGF- β pathway, is consistent with findings that intracellular TGF- β signaling is mainly transduced by Smads (23–26). Our findings support the importance of Smad proteins in viral oncoprotein regulation of TGF- β signaling.

It has been reported that human B lymphocytes expressing vIRF1 were resistant to the antiproliferative effects of IFN- α and that NIH 3T3 cells expressing vIRF1 were refractory to tumor necrosis factor- α -induced apoptosis (19, 51). In addition, vIRF1 inhibited p53-induced apoptosis and IFN/retinoic acid-induced cell death (15, 16, 21, 22). Here, we showed that Mv1Lu cells expressing vIRF1 were resistant to TGF- β -mediated growth arrest due to the targeting of Smad proteins by vIRF1. The strong transforming activity of vIRF1 is likely to be linked to the resistance of vIRF1-expressing cells to various cell death and antiproliferative signals. Tumor suppressor pathways must be inactivated to transform cells and induce tumors. KSHV vIRF1 is a strong oncoprotein, which can transform cells without the aid of any other oncoprotein. Because the TGF- β pathway is a type of tumor suppressor pathway, inhibition of TGF- β can contribute to vIRF1-induced tumorigenicity. Our results allow us to speculate that TGF- β signaling is an important target pathway during viral oncogenesis.

It was initially suggested that vIRF1 was expressed in multicentric Castleman's disease tissue rather than in Kaposi's sarcoma lesions (52). However, a recent report indicates vIRF1 mRNA is present at significant levels in Kaposi's sarcoma and that its transcription profile clustered with KSHV latency-associated nuclear antigen 1 in Kaposi's sarcoma lesions (53). These observations provide evidence that vIRF1 may play a role in Kaposi's sarcoma tumorigenesis. The present data are consistent with the possibility that KSHV vIRF1 inhibition of TGF- β signaling contributes to the development of Kaposi's sarcoma and KSHV-related neoplastic diseases.

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