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Activation of the phosphatidylinositol 3-kinase/Akt pathway by viral interferon regulatory factor 2 of Kaposi's sarcoma-associated herpesvirus

Yejin Kim [1], Seho Cha [1], Taegun Seo*

Department of Life Science, Dongguk University-Seoul, Goyang, 10326, South Korea

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ABSTRACT

Kaposi's sarcoma-associated herpesvirus encodes several genes with sequence homology to cellular interferon regulatory factors. Among these, vIRF2 encoded by ORF K11.1 (short form) or K11 (full-length) participates in caspase-3-mediated inactivation of cellular IRF3 and slightly inhibits caspase-3 activity. Here, we have demonstrated that vIRF2 attenuates the transcriptional activity of forkhead box O3A protein via activation of the PI3K/Akt phosphatidylinositol 3-kinase/Akt pathway, inhibiting FOXO3A-mediated caspase-3 cleavage. Based on the collective findings, we suggest that vIRF2 acts as an activator in PI3K/Akt pathway.

1. Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8, is a member of the gammaherpesvirinae subfamily that causes several malignancies, such as Kaposi's sarcoma, primary effusion lymphoma, and Multicentric Castleman's disease [1–3].

To counteract host immune responses against viral infection, KSHV has evolved several genes that participate in immune evasion strategies. Among these, viral interferon regulatory factors (vIRFs) that share homology with cellular IRFs have been shown to dysregulate interferon (IFN) signaling induced by several cellular IRFs [4–6]. For example, vIRF1 interacts with cellular IRF family members and CBP/p300 to decrease the transcriptional activity of IRFs, leading to disruption of IFN signaling [7].

The first functional study of vIRF2 was performed with the 20 kDa protein encoded by KSHV ORF K11.1 [8]. Transcriptional mapping of vIRFs revealed the existence of another form of vIRF2 encoded by a 2.2 kb transcript. This full-length vIRF2 is translated from two spliced exons of K11.1 and K11 [5,9], and capable of antagonizing the transcriptional activity of IFN-stimulated promoters [5]. Recent studies have suggested that vIRF2 accelerates the caspase-3-mediated degradation of IRF3 and inhibits caspase-3 activity to a limited extent [10], although the exact mechanism is not yet understood.

PI3K is composed of the p110 catalytic subunit and p85 regulatory subunit, and generally activated by intracellular stimuli. For activation of PI3K, the p85 subunit is phosphorylated at a tyrosine residue, releasing it from auto-inhibition [11]. PI3K subsequently migrates to the plasma membrane where it converts phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-triphosphate (PIP_3), which, in turn, interacts with Akt, leading to its translocation to the plasma membrane and phosphorylation.

Phosphorylated Akt regulates a broad range of intracellular proteins that participate in critical cellular events, including cell death, adhesion and proliferation [12–15]. Among the proteins, forkhead family members play central roles in apoptosis [16]. The transcription factor forkhead box O3A (FOXO3A) is predominantly located in the nucleus where it regulates the expression of Fas ligand (FasL) involved in caspase-mediated apoptosis [17]. However, upon phosphorylation by Akt, FOXO3A becomes incapable of inducing FasL transcription, and is exported to the cytoplasm and processed for proteasome-mediated degradation. Consequently, caspase-mediated apoptosis is suppressed. Akt additionally

Abbreviations: vIRF2, viral interferon regulatory factor 2; PI3K/Akt, phosphoinositide 3-kinase/Akt; FOXO3A, forkhead box O3A; ERK, extracellular-regulated kinase.

* Corresponding author. Department of Life Science, Dongguk University-Seoul, 32, Dongguk-ro, Ilsandong-gu, Goyang, Gyeonggi-do, 10326, South Korea.
E-mail address: tseo@dongguk.edu (T. Seo).

[1] These authors contributed equally to this work.

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phosphorylates Raf, leading to inhibition of the Raf/MEK/ERK signaling pathway [18–20].

Here, we have demonstrated that vIRF2 inhibits transcriptional activity of the forkhead response element (FHRE)-containing promoter via activation of the PI3K/Akt signaling pathway. Furthermore, vIRF2 negatively regulates downstream elements of the Akt pathway and abrogates FOXO3A-mediated caspase-3 cleavage. While treatment with the PI3K inhibitor, LY294002, reduced vIRF2-mediated phosphorylation of Akt, the precise mechanism underlying induction of PI3K/Akt signaling remains to be established. Our findings collectively indicate that vIRF2 suppresses FOXO3A-mediated caspase-3 cleavage via a mechanism involving activation of the PI3K/Akt pathway, leading to attenuation of apoptotic responses in KSHV-infected cells.

2. Materials and methods

2.1. Plasmids

To construct the pME18S-vIRF2 vector, we amplified vIRF2 cDNA using total RNA extracts isolated from KSHV-infected BCBL-1 cells. PCR products were subcloned into the *Eco*RI/*Xho*I restriction sites of Flag-tagged pME18S vector. To construct Flag-tagged vIRF2 (1–163) and vIRF2 (1–515), we amplified the individual fragments from pME18S-vIRF2. Fragments were subcloned into the *Eco*RI/*Xho*I (1–163) or *Eco*RI/*Not*I (1–515) restriction enzyme sites of pME18S. FHRE-luc was a gift from Michael Greenberg (Addgene plasmid # 1789) [17]. The GFP-FOXO3A expression plasmid was kindly provided by Dr. Mien-Chie Hung (University of Texas). Constructs encoding AP-1-luc and pRp-luc are described in previous reports [21,22].

2.2. Cells and transfection

293T and NIH3T3 cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin (WelGENE) at 37 °C under 5% CO_2. BJAB cells (both KSHV and Epstein–Barr virus-negative human B cells) were cultured in RPMI1640 (WelGENE) supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C with 5% CO_2. pME18S-vIRF2 (30 μg) were electroporated into BJAB cells in serum-free medium at 210 V and 975 μF. 293T cells were transfected with 4 μg of pME18S-vIRF2 using the standard HBS/$CaCl_2$ method. For caspase-3 cleavage analysis, 293T cells were co-transfected with 3 μg of pME18S-vIRF2 and 1 μg of GFP-FOXO3A plasmids using the standard HBS/$CaCl_2$ method. For immunofluorescence assays, NIH3T3 cells were co-transfected with 1 μg of GFP-FOXO3A and 3 μg of pME18S-vIRF2 expression plasmid using the Convoy transfection reagent (ACTGene). The PI3K inhibitor, LY294002, was purchased from Cell Signaling Technology.

2.3. Luciferase assays

293T cells were co-transfected with 400 ng of pFHRE-Luc and 500 ng of RSV-β-galactosidase (β-gal) plasmids and the indicated amounts of pME18S-vIRF2. Cells were harvested at 24 h post-transfection, and luciferase assays performed using the Luciferase assay system (Promega). β-gal activity was used for normalization of transfection efficiency.

2.4. Western blot and immunoprecipitation

Cells were lysed in radioimmunoprecipitation assay buffer (RIPA; 25 mM Tris–HCl, pH 7.6; 150 mM NaCl; 1% NP-40; 1% sodium deoxycholate; **0.1**% **SDS**) supplemented with 1 mM phenylmethylsulfonylfluoride (PMSF) and phosphatase inhibitor cocktail 2,3 (Sigma). After incubation for 1 h, cells were disrupted with a Bioruptor sonicator (Bio Medical Science; 5 cycles of 30 s on × 30 s off), followed by centrifugation at 12,000 *g* for 30 min at 4 °C. Protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories). Equal amounts of proteins were resolved via 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 3% BSA in TBST [TBS (20 mM Tris–HCl, pH 7.5; 120 mM NaCl) with 0.1% (v/v) Tween 20] for 1 h, and subsequently incubated with the indicated primary and secondary antibodies for 1 h each. Proteins were detected using the chemiluminescent substrate (Thermo Scientific). Relative protein concentrations normalized by β-actin were measured using densitometer software.

For immunoprecipitation, whole-cell lysates were incubated with rabbit anti-p85 (Santa Cruz Biotechnology) for 1 h at 4 °C, followed by precipitation with protein G sepharose 4 Fast Flow (GE Healthcare) beads for 1 h at 4 °C. Proteins were collected by centrifugation at 2000 *g* for 5 min, and subsequently washed three times with RIPA. Precipitates were analyzed via western blot with HRP-conjugated anti-phosphotyrosine antibody (Cell Signaling Technologies).

2.5. Immunofluorescence

At 24 h post-transfection, NIH3T3 cells were fixed with 3.7% formaldehyde for 15 min. Fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min and blocked with 2% BSA for 30 min. Cells were incubated with the indicated antibodies for 1 h and washed with PBS three times for 5 min each, followed by incubation with anti-mouse IgG-Alexa Fluor 555 (Cell Signaling Technology) and anti-rabbit IgG-FITC (Sigma–Aldrich) for 1 h. DNA was stained with 1 μg/ml DAPI (Sigma), and cells were analyzed under a confocal microscope (Eclipse Ti; Nikon).

3. Results

3.1. KSHV vIRF2 inhibits the transcriptional activity of FHRE-containing promoters

To determine whether vIRF2 regulates the cellular signaling pathway similar to other vIRFs, we performed the luciferase assay with various reporter plasmids. We found that vIRF2 inhibited transcriptional activity of the FHRE-containing promoter in a dose-dependent manner (Fig. 1A). Since a number of forkhead box proteins are known to participate in various intracellular metabolism pathways, including DNA repair, apoptosis and multiple pathogenesis, we further focused on the mechanism by which vIRF2 regulates the FHRE-associated pathway. According to the conserved domain database, vIRF2 contains IRF (aa. 7–114) and IRF3 (aa. 516–660) domains at the N and C-terminal regions, respectively. Because we did not observe the sole IRF3 domain expression, we used plasmids expressing peptides encompassing amino acids 1 to 163 and 1 to 515 to determine the functional region inhibiting the transcriptional activity of the FHRE-containing promoter. As shown in Fig. 1B, vIRF2 (1–515), but not vIRF2 (1–163) suppressed transcriptional activity of the FHRE-containing promoter.

Next, we confirmed whether vIRF2 expression affects translocation of FOXO3A to the cytoplasm, with a view to identifying the forkhead box protein that participates in vIRF2-mediated transcriptional regulation. FOXO3A is expressed in various cell types, and translocation to the cytoplasm is a visual marker of protein inactivation [23]. Following co-transfection with the indicated plasmids (Fig. 1C), in cells transfected with empty vector we observed the presence of GFP-FOXO3A throughout cells, whereas it

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