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Gelsolin segment 5 inhibits HIV-induced T-cell apoptosis via Vpr-binding to VDAC

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Abstract **Viral protein R (Vpr) from the human immunodeficiency virus induces cell cycle arrest in proliferating cells, stimulates virus transcription, and regulates activation and apoptosis of infected T-lymphocytes. We report that Jurkat cells overexpressing full-length gelsolin show resistance to Vpr-induced T-cell apoptosis with abrogation of mitochondrial membrane potential loss and the release of cytochrome *c*. Co-immunoprecipitation assays in HEK293T cells demonstrated that overexpression of full-length or segment 5 (G5) but not G5-deleted gelsolin (ΔG5) bound to the voltage-dependent anion channel (VDAC), and that the G5 subunit can inhibit HIV-1-Vpr-binding to VDAC. We also confirmed that full-length gelsolin has the same effect in Jurkat cells. Clonogenic analysis showed that transfection of G5 but not ΔG5 cDNA protects Jurkat T cells from HIV-Vpr-Tet induced T-cell apoptosis and promoted cell survival, as did full-length gelsolin. These results suggest that the gelsolin G5 domain inhibits HIV-Vpr-induced T-cell apoptosis by blocking the interaction between Vpr and VDAC, and might be used as a protective treatment against HIV-Vpr-induced T-cell apoptosis.**

Keywords: Cytoskeletal protein; AIDS; Mitochondria; Cell death

1. Introduction

Multiple mechanisms have been proposed to explain the death and dysfunction of CD4+ T-cells after infection with the human immunodeficiency virus type 1 (HIV-1) [1]. There are various molecular HIV-1 components that play a role in the induction of apoptosis in T-lymphocytes [1]. Viral protein R (Vpr) plays an important role in regulating the nuclear transport of the HIV-1 pre-integration complex, and is required for virus replication in non-dividing cells [2,3]. Vpr also induces cell cycle arrest in proliferating cells, stimulates virus transcription, and regulates activation and apoptosis in infected cells [2,4,5]. These changes occur in the absence of other viral gene products, suggesting that Vpr mediates its proviral effects at least partially or perhaps solely, through modulation of the state of the target cell rather than directly by the virus [3]. Vpr from HIV-1 attaches to mitochondrial membranes and induces mitochondrial membrane permeabilization (MMP), which is a critical step in the regulation of apoptosis and is often accompanied by mitochondrial swelling and fragmentation [6,7].

Gelsolin, an actin-regulatory protein that modulates actin assembly and disassembly, is found as both an intrinsic cytoplasmic protein and secreted plasma protein [8,9]. In addition, gelsolin was identified as a substrate for caspase-3 by screening the translation products of small complementary DNA pools for sensitivity to cleavage by caspase-3 [10]. Expression of gelsolin cleavage product in multiple cell types caused the cells to detach, round up, and undergo nuclear fragmentation [10]. It was proposed that its association with actin drives the calcium-independent activation of the N-terminal three domains gelsolin G1–G3 during apoptosis [11]. Conversely, some reports have previously shown that cytoplasmic gelsolin is also present in the mitochondrial fraction of cells, and that full-length gelsolin can inhibit apoptosis of human Jurkat T-cells [12,13]. The overexpression of gelsolin inhibits the loss of mitochondrial membrane potential and cytochrome *c* release from mitochondria, resulting in a lack of activation of caspase −3, −8, and −9 in Jurkat cells treated with staurosporine, thapsigargin, and protoporphyrin IX [13]. This anti-apoptotic function of gelsolin was also observed in butyrate-induced apoptosis of colorectal cancer cells and the cholinergic toxin ethylcholine aziridinium-or amyloid-beta-induced apoptosis of neuronal cells [14–17], and segment G5 of human cytoplasmic gelsolin is sufficient for the function recorded in the latter case [17].

In this study, our efforts were directed towards investigating whether gelsolin can inhibit HIV-Vpr-induced cell death in Jurkat T cells, and to determine the specific gelsolin domain responsible for that function.

2. Results

2.1. Resistance of gelsolin-overexpressed Jurkat T-cells to HIV-Vpr-induced apoptosis

In an effort to determine whether overexpression of gelsolin in Jurkat T-cells affects HIV-Vpr-induced apoptosis, we used human cytoplasmic gelsolin-stably overexpressed in a Jurkat

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Abbreviations: HIV-1, human immunodeficiency virus type 1; Vpr, viral protein R; VDAC, voltage-dependent anion channel; MMP, mitochondrial membrane permeabilization; IB, immunoblotting; Dox, doxycycline; PTPC, permeability transition pore complex; ANT, adenine nucleotide translocator; NMDA, *N*-methyl-D-aspartate; VDCC, voltage-dependent calcium channels; rtTA, reverse tetracycline-controlled transactivator; PI, propidium iodide; IP, immunoprecipitation

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T-cell clone JGF that was previously established [12]. To confirm continuous expression of gelsolin in JGF and Neo control clone JNF cells, immunoblotting (IB) analysis was performed. Gelsolin was not detected in JNF, while JGF displayed expression of gelsolin (90 KD) and was in keeping with previous results (Fig. 1A). The level of actin remained unchanged among all cell lines. Since we were not able to acquire HIV-Vpr stable transfectants using a constitutively expressing Vpr plasmid, we used an inducible expression system based on the tetracycline-responsive operon (Tet-on system) [18]. Jurkat Tet-on cell lines were created as described in Section 4 below that stably express FLAG-tagged HIV-Vpr by selection in medium containing hygromycin and zeocin. Several clones derived from cells transfected with HIV-Vpr were isolated, analyzed by IB analysis using anti-FLAG antibody to confirm the high expression of HIV-Vpr, and HIV-Vpr-overexpressed parental clone (JPV 5), JNF clones (JNFV 3, 4, 7) , and JGF clones (JGFV 2, 8, 9) were established. JP cells and all clone cells did not express HIV-Vpr under basic conditions (Fig. 1B). However, treatment with doxycycline (Dox) for 48 h resulted in a marked increase of recombinant Vpr protein expression without any detectable change in β-actin expression in parental cells and all clones. At different time points after Dox addition, JPV clone 5, JNFV clones (3, 4, 7) and JGF clones (2, 8, 9) were assayed for cell viability and apoptosis signs using Hoechst 33342 and PI staining. Hoechst 33342 staining showed HIV-Vpr-induced apoptosis accompanied by changes in nuclear morphology, such as nuclear condensation or fragmentation in control JNFV clones at 24, 48, 72 h after Dox treatment, while JGFV clones treated with Dox expressing HIV-Vpr failed to show any morphological nuclear changes (Fig. 2A). Cell viability analysis using Hoechst 33342 and PI revealed that all JGFV clones were clearly more resistant to apoptosis induced by Vpr compared to JPV clone 5 cells and all JNFV clones after 24 h (Dead cells: JGFV, 5% to JPV, 24%, JNFV, 23%), 48 h (Dead cells: JGFV, 9% to JPV, 46%, JNFV, 44%) and 72 h (Dead cells: JGFV, 12% to JPV, 55%, JNFV, 54%) (Fig. 2B) after initial Dox treatment. These data demonstrated that overexpression of gelsolin was associated with significant resistance to HIV-Vpr-induced apoptosis.

2.2. Inhibition of mitochondrial membrane potential loss and cytochrome c release stimulated with HIV-Vpr in gelsolin-overexpressed Jurkat T cells

Other report demonstrated that gelsolin can inhibit apoptosis induced by several apoptotic reagents by blocking signal transduction at the mitochondrial level upstream of the caspase cascade in human T lymphocytes [13]. To analyze the alteration in DeltaPsi(m) that follows HIV-Vpr apoptotic stimulation, we incubated cells after Dox treatment with the cationic dye Rhodamine 123 and then analyzed the cells using a flow-cytometer. JPV clone 5 and all JNF clones displayed loss of all mitochondrial potential, while JGFV clones demonstrated inhibitory activity (Fig. 3A). Another change observed in the mitochondria of apoptotic cells is the translocation of cytochrome *c* from within the innermost mitochondrial membrane to a cytosolic location. Immunoblot analysis of cytosolic fractions revealed that JPV clone 5 and all JNF clones showed cytochrome *c* release after Dox treatment, while there was almost no cytochrome *c* release from the JGF clones (Fig. 3B). Evaluation of all the data indicates that gelsolin

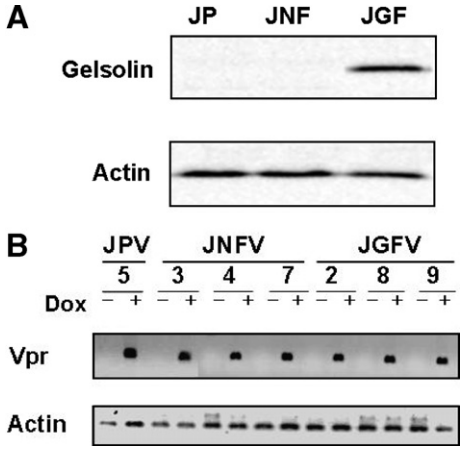

Fig. 1. Expression of gelsolin and HIV-Vpr in Jurkat cell lines by IB analysis. (A) Confirmation of stable expression of human full-length cytoplasmic gelsolin in Jurkat T cell line (JGF). JP, parental Jurkat cells; JNF, neo-transfected Jurkat clone. (B) Inducible expression of Flag-tagged HIV-Vpr in Jurkat Tet-on clones (JPV clone 5, JNFV clones 3, 4, 7, and JGFV clones 2, 8, 9) at 24 h in the absence or presence of 2 μg/ml Dox. The expression of actin was monitored to ensure equivalent loading and transfer.

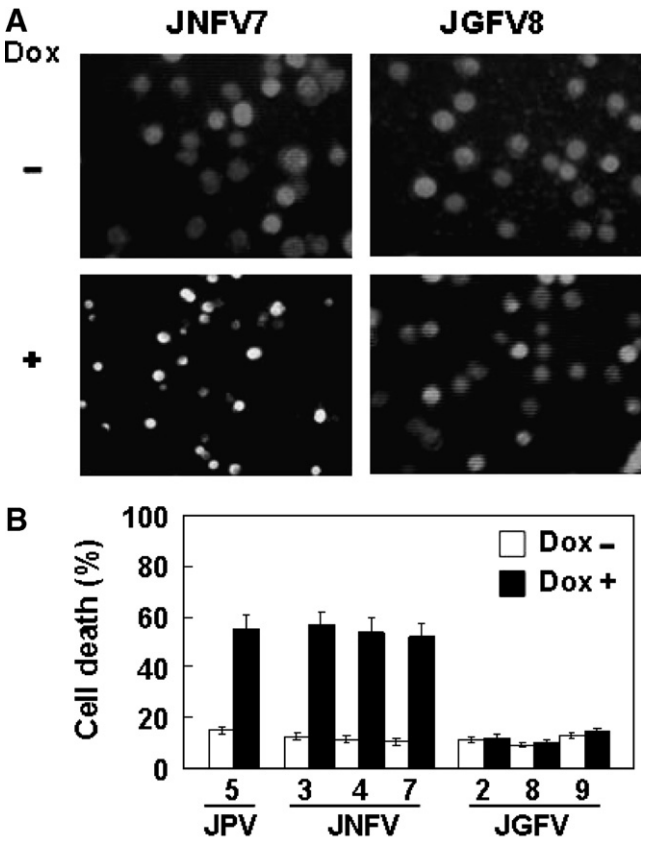

Fig. 2. Cell viability in HIV-Vpr expressed Jurkat cell lines. (A) Hoechst staining of HIV-Vpr expressed JNFV7 and JGFV8 cells at 72 h in the presence or absence of 2 μg/ml Dox. (B) Cell viability of JPV, JNFV, and JGFV cell lines at 72 h in the presence or absence of 2 μg/ml Dox was calculated as the percentage of apoptotic cells compared to total cells using Hoechst 33342 and PI. Three experiments were performed in duplicate, and values represent the mean + S.E. of dead cells.

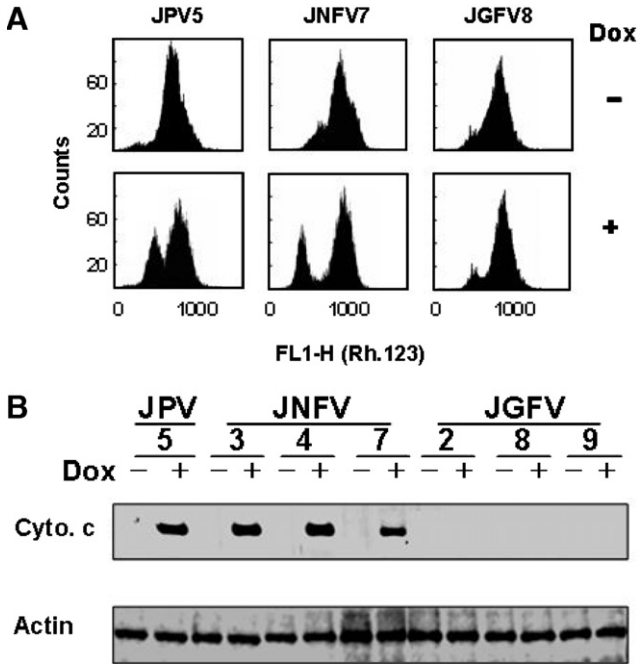

Fig. 3. MMP and cytochrome *c* release from mitochondria in HIV-Vpr expressed Jurkat cell lines. (A) MMP assessed by flow-cytometry with Rhodamine 123 of JPV5, JNFV7, and JGFV8 cells at 72 h in the presence or absence of 2 μg/ml Dox. (B) Cytochrome *c* release from mitochondria of JPV, JNFV, and JGFV cell lines at 72 h in the presence or absence of 2 μg/ml Dox.

can effectively inhibit HIV-Vpr-induced apoptosis at a point concomitant with, or upstream of, the mitochondrial events.

2.3. Inhibition of HIV-Vpr binding to VDAC by full-length gelsolin and gelsolin segment 5 (G5)

We have previously shown that segment 5 of gelsolin (G5) represents an important regulatory region in determining its inhibitory effect upon cell apoptosis [17]. Recent reports suggested that VDAC is a key molecule controlling apoptotic mitochondrial changes and the HIV-Vpr induces apoptosis via a direct effect on the mitochondrial permeability transition pore complex (PTPC) by binding VDAC [6]. To verify the interaction between full-length gelsolin, G5, and VDAC, co-immunoprecipitation experiments were performed. HEK293T cells were transiently transfected with an expression plasmid encoding Myc-tagged full-length gelsolin, G5, or G5-deleted gelsolin (ΔG5) together with expression plasmid encoding T7-tagged VDAC, and the cell lysates were immunoprecipitated with anti-Myc antibody. The resulting precipitates and a portion of the cell lysate were subjected to IB analysis with anti-T7 tag and anti-Myc antibodies. Results of IB analysis showed that VDAC coprecipitated with full-length gelsolin and the G5 domain, but not with ΔG5 transfectant, suggesting that gelsolin is physically associated with VDAC and that the G5 segment of gelsolin is necessary and by itself sufficient for this interaction (Fig. 4A).

The observed interaction allowed us to determine if gelsolin and HIV-Vpr compete for the binding of VDAC in cultured cells. HEK293T cells were transiently transfected with expression plasmids encoding Flag-tagged Vpr and T7-tagged VDAC, together with increasing amounts of expression plasmid for Myc-tagged G5 (Fig. 4B). Cell lysates were prepared from transfected cells, immunoprecipitated with anti-T7 tag antibody, and the resulting precipitates and a portion of cell lysate were subjected to IB analysis with anti-FLAG, anti-T7 tag and anti-Myc antibodies. The results of IB analysis showed that VDAC-bound-Vpr was reversibly reduced with the amount of G5 administered in a dose-dependent manner. The amounts of Vpr and VDAC expressed were almost constant. These results indicate that G5 blocks the interaction between HIV-Vpr and VDAC.

Therefore, we also examined the effect of full-length gelsolin on the physical interaction between Vpr and the VDAC in

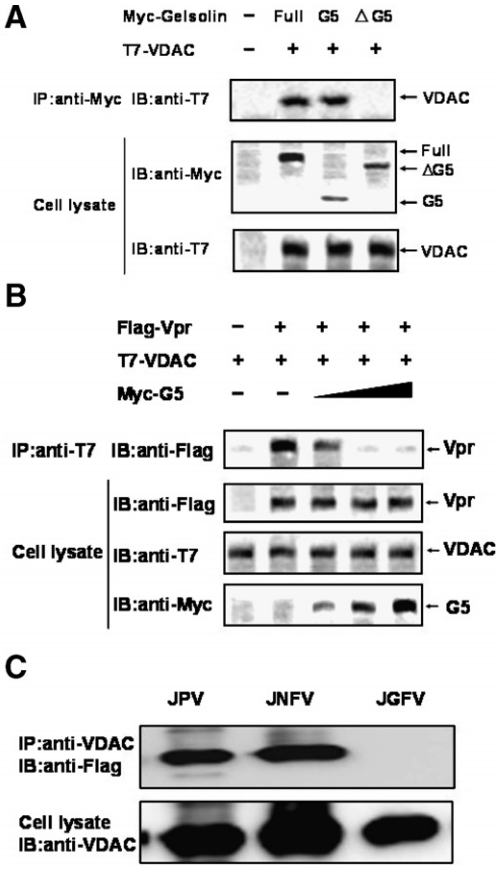

Fig. 4. Inhibition of VDAC-Vpr interaction by full-length gelsolin and the G5 segment of gelsolin. (A) HEK293T cells were transiently transfected with Myc-tagged full-length gelsolin (Full), G5 or ΔG5 (1 μg) together with T7-tagged VDAC (1 μg) as indicated. Cell lysates were subjected to immunoprecipitation (IP) with anti-Myc antibody, and the resulting precipitates were subjected to IB with anti-T7 tag antibody. A portion of the cell lysate was directly subjected to IB with anti-Myc, anti-T7 tag antibody in order to verify the expression level of gelsolin and VDAC proteins. (B) HEK293T cells were transfected with Flag-tagged HIV-Vpr and T7-tagged VDAC together with increasing amounts of Myc-G5 (0.5, 1, and 2 μg). Cell lysates were subjected to IP with anti-T7 tag antibody, and the resulting precipitates were subjected to IB with anti-FLAG antibody. A portion of the cell lysate was directly subjected to IB with anti-FLAG, anti-T7 tag, and anti-Myc antibodies to verify the expression level of Vpr, VDAC, and G5 proteins. (C) JPV, JNFV and JGFV cells were treated with Dox for 48 h. Cell lysates were subjected to IP with anti-VDAC antibody, and the resulting precipitates blotted with anti-Flag antibody. A portion of the cell lysate was directly subjected to IB with anti-VDAC in order to verify the VDAC protein expression level (internal control).

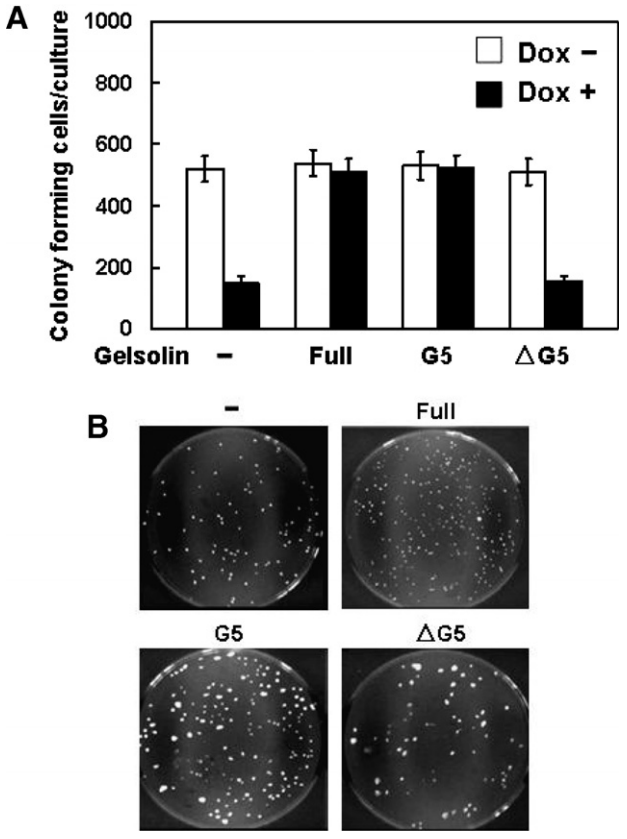

Fig. 5. G5 segment allows Dox-treated HIV-Vpr Tet-on Jurkat T cells to form colonies. (A) Cells were assayed for colony-forming ability 2 weeks after apoptotic induction by Dox treatment with the transfection of an empty plasmid (–), full-length gelsolin (Full), G5 or ΔG5 expression plasmids. Means and standard errors of two independent experiments each containing three replicates are shown. (B) Plates are illustrated below the corresponding Dox (+) columns of the histogram.

Jurkat cells, using an immunoprecipitation assay system (Fig. 4C). Cells were treated with Dox after 48 h, cell lysates were immunoprecipitated with anti-VDAC and the immunocomplexes probed with Flag tagged Vpr or gelsolin antibodies. These results showed that Vpr can interact with VDAC in JPV and JNFV cells, but this interaction cannot be detected in JGFV cells. These results demonstrate that Vpr is bound to VDAC in JPV clones and all JNFV clones, but this interaction was inhibited by full-length gelsolin overexpressing Jurkat cells after Dox treatment.

2.4. Protective function of G5 from HIV-Vpr-induced apoptosis in Jurkat T cells

In order to determine the protective function of full-length gelsolin and G5 from HIV-Vpr-induced apoptosis in the Jurkat cell line, we examined their effects by utilizing the most stringent criterion for cell survival: the ability to form a colony from only a single cell. Treatment of Dox in parental Jurkat HIV-Vpr Tet-on (JPV) clone 5 cell line with an empty control plasmid produced a substantial reduction in colonies that subsequently formed in soft agar (Fig. 5A and B). The transfection of expression plasmids with full-length gelsolin or G5, however, produced a 3.4-fold and 3.6-fold respective increase in the number of colony-forming cells surviving this treatment in the JPV clone 5 cells, while that of an empty or ΔG5 plasmids showed no such increase (Fig. 5A and B). These results indicate that the G5 domain and not ΔG5 has a protective function against HIV-Vpr-induced apoptosis in Jurkat T cells, similar to full-length gelsolin.

3. Discussion

HIV-Vpr causes a rapid dissipation of mitochondrial membrane potential, as well as a mitochondrial release of apoptogenic proteins such as cytochrome *c* or apoptosis inducing factor [6]. The effects of both mitochondrial and cytotoxic Vpr are prevented by Bcl-2, an inhibitor of the permeability transition pore complex (PTPC) [19]. Vpr favors the permeabilization of artificial membranes containing purified PTPC or defined PTPC components such as the adenine nucleotide translocator (ANT) combined with Bax. Again, this effect is prevented by the addition of recombinant Bcl-2. The Vpr carboxyl terminus binds purified ANT, as well as a molecular complex containing ANT and VDAC, another PTPC component. Vpr induces apoptosis via a direct effect on the mitochondrial PTPC [6,19].

Koya et al. previously reported that human cytoplasmic gelsolin is localized not only in the cytosol, but also in the mitochondrial fraction of cells, and that it inhibits the loss of DeltaPsi(m) and cytochrome *c* release from mitochondria in Jurkat cells treated with staurosporine, thapsigargin and protoporphyrin IX [13]. Furthermore, overexpression of gelsolin inhibits the loss of DeltaPsi(m) and cytochrome *c* release from mitochondria and inhibits activation of caspase −3, −8, and −9 in Jurkat cells treated with staurosporine, thapsigargin, and protoporphyrin IX. These effects were corroborated in vitro using recombinant gelsolin protein on isolated rat mitochondria stimulated with calcium, atractyloside, or Bax [13]. The carboxyl-terminal half of gelsolin may also prevent apoptotic mitochondrial changes such as DeltaPsi(m) loss and cytochrome *c* release in isolated mitochondria and inhibit the activity of VDAC on liposomes [20]. Segment 5 of human cytoplasmic gelsolin is the region responsible for inhibition of Amyloid beta-induced cytotoxicity in PC12 rat neuronal cells, in addition to full-length gelsolin [17]. Primary hippocampal neurons cultured from mice lacking gelsolin showed enhanced calcium influx after exposure to glutamate [15]. Whole-cell patch-clamp analyses showed that currents through *N*-methyl-D-aspartate (NMDA) receptors and voltage-dependent calcium channels (VDCC) were enhanced in hippocampal neurons lacking gelsolin. These results suggest roles for gelsolin in events that involve activation of NMDA receptors and VDCC [15]. In this report, we have uncovered another function of gelsolin associated with the ion channel by demonstrating binding to VDAC in mitochondria.

Here we report that gelsolin inhibits HIV-Vpr-induced apoptosis accompanied by the loss of DeltaPsi(m) and cytochrome *c* release from mitochondria in Jurkat T cells. We took advantage of the high transfection efficiency of HEK293T cells to study interactions between VDAC and gelsolin, and demonstrated binding between full-length gelsolin and the G5 segment, but not between other segments and VDAC. Moreover, we demonstrated in HEK293T cells that gelsolin segment 5 shows overlapping, competitive binding of VDAC with HIV-Vpr. These results suggest that gelsolin G5 domain inhibits

HIV-Vpr-induced T-cell apoptosis by blocking binding between Vpr and VDAC. In addition, similar results were obtained in Jurkat cells using full-length gelsolin.

There are reports showing that the presence of antioxidants, such as *N*-acetyl-cysteine, nicotinamide or L-acetyl-carnitine, were able to rescue most of the peripheral blood lymphocytes of subjects with acute HIV syndrome from apoptosis through a protective effect on mitochondria [21]. Additionally, IL-2 and IL-4 produced by peripheral blood mononuclear cells during highly active retroviral therapy provided anti-apoptotic signals that may contribute to an increased survival of T-cells and may thus play a part in long-term immune reconstitution [22]. In this report, we have demonstrated significant protection produced by the G5 gelsolin subunit, but not with other gelsolin domains, from HIV-Vpr-induced apoptotic induction of Jurkat T cells grow on soft agar. These findings suggest a rationale for the use of gelsolin segment 5 protein treatment, in addition to antiviral drugs, in primary HIV infection.

4. Materials and methods

4.1. Plasmid construction

To construct a mammalian expression plasmid with Myc-tagged human cytoplasmic gelsolin (pCI-neo-6xMyc-Gelsolin), the coding sequence was amplified by PCR using LKCG (a kind gift from D. Kwiatkowski of Harvard Medical School) as a template. The resulting PCR products were subcloned into the *Eco*RI–*Sal*I site of the pCI-neo-6xMyc mammalian expression plasmid [23]. Two gelsolin mutants, G5 that encodes segment 5 of human gelsolin (amino acids 516–618) and ΔG5 that lacks the region encoding segment 5, were generated using a PCR-based method and the resulting PCR products were subcloned into pCI-neo-6xMyc, and pCI-neo-6xMyc-G5 and pCI-neo-6xMyc-ΔG5 were prepared, respectively. Human VDAC1 cDNA was obtained by RT-PCR using forward primer (5′-TATGAATTCATGTGTAACACACCAACG-3′) and reverse primer (5′-TATCTCGAGCCTCAAACCACATTAAGC-3′) [24], and the resulting PCR product was subcloned into the *Eco*RI–*Sal*I site of pCI-neo-3xT7 as described previously [23], and pCI-neo-3XT7-VDAC was constructed. Vpr sequence derived from the plasmid vector pME18Neo-F(lag)Vpr containing HIV-1-Vpr [5] (a generous gift from Dr. Aida, Retrovirus Research Unit, RIKEN, Wako, Saitama, Japan) and the Flag tag-Vpr was subcloned into plasmid vector pTRE2-Hyg (Clontech) to generate the pTRE2-Hyg-Vpr plasmid.

4.2. Cell culture and establishment of stable cell lines

A lymphoblastoid T-cell line Jurkat (parental Jurkat: JP), and its stable clones transfected with human cytoplasmic gelsolin plasmid LKCG (JGF clone 5) or with the control plasmid LK444 (a kind gift from P. Gunning) (JNF clone 2), were maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS) (Gibco BRL, Gaithersburg, MD) as described previously [12]. HEK293T cells was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS. All cells were cultured at 37 °C in a 5% CO2 humidified atmosphere.

The Tet-on system (Clontech, Mountain View, CA) was used to obtain stable cell lines that express HIV-1-Vpr. JP, JNF and JGF (1×10^6) were transfected with 2 μg of regulator plasmid pTracer-CMV2-Tet-on containing reverse tetracycline-controlled transactivator (rtTA), which interacts with the inducible promoter in the presence of tetracycline or analogues as Dox and activates transcription, and 12 μg/ml of SuperFect transfection reagent (Qiagen, Tokyo) according to the manufacturer's instructions. Each transfectant was selected in the presence of 0.8 mg/ml zeocin (Invitrogen, Carlsbad, CA). To select clones with high expression of rtTA, total RNA was isolated from JP-Tet-on JNF-Tet-on and JGF-Tet-on cell lines with extraction reagent (TRIzol, Invitrogen). The RT-PCR was performed with 1 μg of RNA from each sample, with reverse transcriptase (Superscript II; Gibco BRL, Carlsbad, CA) and random primer. The reverse transcript was incubated with Taq-polymerase and primers rtTA forward (5′-GAGGTCGGAATCGAAGGTTT-3′), which matches the coding strand of rtTA at positions 55–74, and rtTA reverse (5′-TCGTAATAATGGCGGCATAC-3′), which matches the reverse strand of rtTA at positions 513–522, as described previously [24], for 35 cycles (denaturing: 30 s 95 °C; annealing: 30 s 55 °C; elongation: 90 s 72 °C), followed by a 7 min 72 °C extension. Electrophoresis of PCR-products was performed on a 1% agarose gel containing ethidium bromide. JP-Tet-on JNF-Tet-on and JGF-Tet-on cell lines that expressed higher rtTA were selected. Next, the selected Tet-on cell lines, JP-Tet-on JNF-Tet-on and JGF-Tet-on, were transfected with pTRE2-Hyg-Vpr and each transfectant (JPV clone 5; JNFV clones 3, 4, 7; JGFV clones 2, 8, 9) was further selected in the presence of 400 μg/ml Hygromycin (Wako, Osaka, Japan). To investigate clones with high expression of HIV-Vpr, each Tet-on cell line was treated with 2 μg/ml of Dox and the expression of HIV-Vpr confirmed by IB.

4.3. Immunoblotting analysis

Total cells were extracted in SDS sample buffer (40 mM Tris–HCl, pH 7.4, 5% 2ME, 2% SDS, 0.05% bromphenol blue). Cell lysates were analyzed by SDS–polyacrylamide gel electrophoresis and IB as described previously [13,22] using monoclonal anti-human gelsolin (GS-2C4, Sigma), monoclonal anti-cytochrome *c* (Pharmingen, Mississauga, ON, Canada), anti-Myc monoclonal antibody (Clontech), anti-T7 tag monoclonal antibody (Novagen, San Diego, CA), monoclonal anti-FLAG (M2) antibody (Sigma), monoclonal anti-VDAC (Sigma) and anti-β-actin monoclonal antibody (Chemicon, Temecula, CA). The bound primary antibodies were incubated with peroxidase-conjugated anti-mouse IgG+M (Jackson ImmunoResearch Lab., West Grove, PA) and detected by ECL Western blotting detection reagents (Amersham Biosciences). Band images were detected by a LAS 1000 mini system (Fuji Film, Kanagawa, Japan).

4.4. Assays for cell viability and mitochondrial functions

Cell viability and apoptotic cell death were assessed using Hoechst 33342 (Sigma, St. Louis, MO) and propidium iodide (PI) staining, MMP assayed by the addition of Rhodamine 123 to the culture medium, and cytochrome *c* release from mitochondria into the cytosol of Jurkat cells evaluated by SDS–polyacrylamide gel electrophoresis followed by IB of the cytosolic fraction, as previously described [13]. For cell viability and mitochondrial functions, JPV, JNFV, and JGFV cell lines were examined at 24, 48 or 72 h and 72 h in the presence or absence of 2 μg/ml Dox, respectively.

4.5. Co-immunoprecipitation analysis

HEK293T cells were transiently transfected with expression plasmid as indicated. Forty-eight hours after transfection, the cells were washed with ice-cold Tris-buffered saline (TBS) and harvested. The cells were then lyzed with immunoprecipitation (IP) buffer containing 50 mm Tris–HCl (pH 7.5), 150 mm NaCl, 0.5% Triton X-100, 10% glycerol, 0.1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml chymostatin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. The lysates were incubated on ice for 30 min, and the cell debris was removed by centrifugation at 13000*g* for 20 min. The resulting supernatants were pretreated with 20 μl Protein G-Sepharose beads (Roche,Tokyo, Japan) at 4 °C for 1 h and was then incubated with 2 μg anti-T7 tag or anti-Myc monoclonal antibody and 20 μl Protein G-Sepharose beads at 4 °C for 2 h. The immunocomplex that was produced was washed five times with IP buffer. SDS-sample buffer was added to the beads, and the samples were boiled. The immunoprecipitates and the cell lysates were subjected to IB analysis. For JPFV, JNFV and JGFV cell lines, 48 h after treatment with Dox, the cell lysates were then incubated with 2 μg anti-VDAC monoclonal antibody under the same conditions as previously mentioned.

4.6. Clonogenic assay

Clonogenic analysis was performed as described previously [25]. Using electroporation, 1×10^7 JPV clone 5 cells were transfected with expression plasmids: pCI-neo-6xMyc, pCI-neo-6xMyc-Gelsolin, pCI-neo-6xMyc-G5 or pCI-neo-6xMyc-ΔG5. Forty-eight hours after transfection, both 2 μg/ml Dox and 1 mg/ml neomycin were added to the medium. All cells harvested from each plate were suspended in 5 ml of 0.5% agarose containing 20% FCS medium and then plated on the top of 5 ml of 1% semi-solidified agarose (Nacalai Tesque Inc. Kyoto. Japan) with the same medium in 10 cm plates. For each

vector control, full-length gelsolin, G5 and ΔG5 clones, triplicate plates were used. The plates were incubated for 2 weeks at 37 °C in the presence of 5% CO2 in an incubator. They were then stained with 0.5 ml of 0.005% Crystal Violet for more than 1 h. Colonies grown on agarose were counted using a microscope.

4.7. Statistical analysis

The data shown represent mean values of at least three different experiments, expressed as mean ± S.E. Student's *t* test was used to compare the data, and a *P* value of less than 0.05 was considered statistically significant.

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