Human Gyrovirus-Apoptin Interferes with the Cell Cycle and Induces G2/M Arrest Prior to Apoptosis

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Received: 7 October 2016 / Accepted: 20 February 2017 / Published online: 6 April 2017
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Abstract The human gyrovirus-Apoptin (HGyv-Apoptin) is a protein that gained attention because it is selectively cytotoxic toward cancer cells. In this study, we have investigated the effect of HGyv-Apoptin on cell cycle progression of cancer cells. We also compared HGyv-Apoptin's action to its homologue chicken anemia virus Apoptin (CAV-Apoptin). We show that HGyv-Apoptin induces G2/M arrest in cancer cells. This is at least in part due to the fact that HGyv-Apoptin induces an abnormal spindle formation in mitotic cells that do not progress properly throughout the cell cycle. HGyv-Apoptin most likely inhibits APC function leading to a sustained cyclin-B1-expression. These results indicate that HGyv-Apoptin has a similar mechanism of action as its homolog CAV-Apoptin and further supports its cancer therapeutic potential.

Keywords Apoptin · PKTCs · Cell cycle · APC · Cyclin B1

Abbreviations

APC	Anaphase promoting complex
APC/C	Anaphase promoting complex/cyclosome
CAV	Apoptin, chicken anemia virus apoptin
CDK	Cyclin-dependent kinase
GFP	Green-fluorescent protein
Gyv-Apoptin	Human gyrovirus-Apoptin
PKTCs	Proteins killing tumor cells
PML	Promyelocytic leukemia

Introduction

Apoptosis is a form of cell death, which is essential for normal development and for disease control via eliminating the defective cells (Los et al. 1999; Teodoro and Branton 1997). While apoptosis is highly regulated in normal tissue, most of the cancerous tissues show a deregulated apoptotic process due to the mutation of genes indispensable for apoptosis (Pettigrew and Cotter 2009), or genomic stability (Wang et al. 1997; Wiechec 2011). This enables the replication and proliferation of defective cells. While current/old therapies rely on the targeting of one apoptotic abnormality and lack specificity, some of the newly emerged therapies aim the selective targeting of cancer tissues by the so-called PKTCs (proteins killing tumor cells) which are a family of proteins with cancer selective toxicity (Bruno et al. 2009; Rollano Penaloza et al. 2014).

Most of the PKTCs especially the viral PKTCs play a major role in cell cycle progression. The cell cycle progression is orchestrated by checkpoints at two major phases, the S-phase during which the DNA duplication occurs, and the

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M-phase during which the distribution of the genetic material into the two daughter cells takes place (Malumbres and Barbacid 2009). S-phase is preceded by a gap (G1), during which the cell prepares for DNA synthesis. The G1-checkpoint makes sure the internal and external conditions are right for division; this includes the cell size, availability of nutrients, positive molecular signals and DNA integrity. The S-phase is followed by a gap (G2) during which the cell prepares for mitosis. Prior to mitosis, the G2-checkpoint insures that DNA is completely replicated, that replications errors have been repaired, and that the cell is large enough to divide. During mitosis, the spindle assembly check point (SAC) ensures the fidelity of chromosome segregation to prevent chromosome mis-segregation and aneuploidy (Malumbres and Barbacid 2009). SAC is activated during prometaphase to ensure the fidelity of DNA replication. Activated SAC proteins inhibit the ability of anaphase promoting complex/cyclosome (APC/C) cdc20 to ubiquitinate two substrates: securin and cyclin B (Hwang et al. 1998; Kim et al. 1998). Securin inhibits separase, the enzyme that cleaves the cohesion between the sister chromatids and is thus required for the progression of cell division to anaphase (Panigrahi et al. 2008; Peters 2006). While cyclin B forms a complex with cyclin-dependent kinase 1 (CDK1) whose deactivation is essential for mitotic progression and exit (Peters 2006).

The adenoviral protein E4orf4 induces G2/M arrest and apoptosis in cancer cells in a p53-independent manner (Kornitzer et al. 2001). In addition to E4orf4, CAV-Apoptin, another avian-derived viral PKTC, also induces G2/M cell cycle arrest and apoptosis in a p53-independent manner in human malignant cells, while having no deleterious effect on normal cells. CAV-Apoptin shows a differential behavior in transformed and in primary cells. In cancer cells, CAV-Apoptin is phosphorylated on Threonine 108 and accumulates in the nucleus while it remains in the cytoplasm of normal cells, where it is degraded by the proteasome. The C-terminal end of CAV-Apoptin contains a nuclear localization sequence (NLS) and a CRM1-binding nuclear export sequence that

are crucial for the nuclear export of CAV-Apoptin in the cells (Fig. 1). The nuclear localization of CAV-Apoptin is required for induction of apoptosis, which suggests that the cellular target of CAV-Apoptin is/are nuclear protein(s). CAV-Apoptin was shown to interfere with APC1, a subunit of APC/C. This interaction only occurs in cancer cells but not in normal cells. Domains that overlap with Apoptin NLS mediate CAV-Apoptin binding to APC1. This interaction disrupts the APC/C, via the nuclear sequestration of APC1, and maintains an active SAC leading to G2/M cell cycle arrest and apoptosis in a p53-independent manner (Teodoro et al. 2004).

A homolog of chicken anemia virus Apoptin (CAV-Apoptin), human gyrovirus-Apoptin (HGyv-Apoptin) also induces apoptosis selectively in cancer cells and functions independently from p53 (Chaabane et al. 2014; Rollano Penaloza et al. 2014). Little is known about the molecular mechanism of action of HGyv-Apoptin. HGyv-Apoptin has the same subcellular distribution as the CAV-Apoptin; it localizes in the nuclei of cancer cells where it shows a granular distribution that later clusters to form aggregates while it remains in the cytoplasm of normal cells (Bullenkamp et al. 2012). Despite a low overall identity important regions such as the nuclear localization and export signals and phosphorylation sites are conserved between HGyV and CAV-Apoptin suggesting a similar mechanism of action (Sauvage et al. 2011) (Fig. 2).

The purpose of this study is to investigate whether HGyv-Apoptin has a similar effect on cell cycle progression as its homolog CAV-Apoptin and functions via targeting the cell cycle, thus inducing G2/M arrest in cancer cells independently from p53. To this end, we have investigated the effect of HGyv-Apoptin on cell cycle progression. We show that HGyv-Apoptin induces abnormal spindle and G2/M arrest in cancer cells. HGyv-Apoptin seems to interfere with APC/C function, as cells expressing HGyv-Apoptin maintain high levels of CyclinB1 in cancer cells but not in normal cells, thus preventing the completion of the cell cycle and the mitotic exit.

Fig. 1 Diagrammatic representation of CAV-Apoptin functional domains (the number of aminoacids at each structural motif is indicated). *PML* promyelocytic leukemia, *NES* nuclear export sequence, *NLS* nuclear localization sequence, *LRS* leucine-rich sequence

Fig. 2 Sequence alignment of CAV- and HgyV-Apoptin (<http://xylian.igh.cnrs.fr/bin/align-guess.cgi>). Important functional domains, including LRS, NLS1/2 and nuclear export sequence (NES) are indi-

cated by *boxes* and the predicted phosphorylation sites threonine 108 or threonine 111 are indicated by *black arrows*

Materials and Methods

Antibodies

The following primary antibodies were used: mouse anti- α -actin (Abcam, UK), α/β -Tubulin Antibody (Cell signaling), Anti-cyclin B1 (Millipore, Germany), Anti-phospho-Ser/Thr-Pro MPM-2 Antibody (Millipore, Germany). The following secondary antibodies were used: Infrared dye 800cw goat, anti-rabbit antibody, Infrared dye 680cw goat, anti-mouse antibody (Licor, USA), Rhodamine redX anti-rabbit antibody (life technologies), cy5 anti-murine antibody (Abcam, UK).

Cell Culture and Reagents

HCT116 (colon carcinoma) cells were grown in DMEM medium supplemented with 10% fetal calf serum (Hyclone), 100 μ g/ml penicillin and 0.1 μ g/ml streptomycin (Gibco BRL, New Zeland). Cells were cultured at 37 °C with 5% CO₂ in a humidified incubator.

Plasmids and Transient Transfections

The following expression plasmids were used: GFP-HGyv-apoptin (GFP-HGV-APT), GFP-CAV-apoptin (GFP-CAV-APT), and the negative control pEGFPC1. Cells were transfected with pEGFPC1 vector alone or with GFP-HGV-APT and GFP-CAV-APT using XtremeGENE HP DNA Transfection Reagent according to the manufacturer's instructions (Roche, Germany).

Cell Extracts and Immunoblotting

The level of protein expression of cyclin B1 was detected by immunoblotting. Briefly, 2 $\times$ 10⁵ cells were transfected with pEGFPC1 vector alone or with GFP-HGyv-apoptin. The expression of GFP and GFP-HGV-APT was confirmed by fluorescence microscopy. At the indicated time periods, whole cell lysates were prepared using RIPA Buffer and according to the manufacturer's instructions (Thermo-scientific). Proteins (30 μ g) were separated by denaturing SDS-PAGE with 30% T acrylamide-Bisacrylamide and then transferred onto a PVDF membrane. The membranes were blocked in 5% non-fat dried milk in TBS 0.1% tween and then incubated overnight with the following primary antibody rabbit anti-cyclin B1 (Millipore, Germany), and mouse anti- β -actin (Abcam, UK) at 4 °C. Then, the blots were incubated with the corresponding InfraRed dye secondary antibodies (LICOR). Visualization was carried out by a LICOR membrane scanner.

Immunocytochemistry and Confocal Imaging

Cells were grown overnight on coverslips and then transfected either with GFP-HGV-APT, GFP-CAV-APT or pEGFPC1. At the indicated time points, cells were washed with PBS and then fixed in 4% paraformaldehyde for 20 min (Cao et al. 2016; Heilman et al. 2006; Teodoro et al. 2004). Cells were then permeabilized with 0.1% Triton X-100 and blocked in 0.1% BSA. Cells were incubated with α/β -Tubulin antibody overnight at 4 °C, followed by three wash steps with PBS and 1 h incubation with the

corresponding Rhodamine redX secondary antibody. The nuclei were visualized by DAPI staining. The fluorescent images were then observed and analyzed with a Zeiss LSM 510 inverted laser-scanning confocal fluorescence microscope using 40X oil planochromat objective (Carl Zeiss, Thornwood, NY).

Cell Cycle Analysis

Cells were washed in PBS, fixed in 70% ethanol and stored at 4 °C for 1 h. Cells were then resuspended in 1% FCS in PBS and stained with 50 µl MPM-2 antibody overnight at 4 °C. Next morning, cells were washed thrice in PBS. And then stained with anti-cy5 secondary antibody for 1 h at RT, cells were then washed thrice in PBS and resuspended in PBS containing 40 µg/ml of propidium iodide (PI) for 30 min followed by Analysis by flowcytometry using FL1 for FITC, FL4 for cy5 and FL3 for PI.

Results

HGyv-Apoptin Induces G2/M Arrest Selectively in Cancer Cells

We first checked the effect of HGyv-Apoptin on cell cycle progression. For this purpose, HCT116 cycling cells were transfected with GFP-HGyv-apoptin; pEGFPC1 was used as a negative control while GFP-CAV-apoptin was used as a positive control. The cells were stained with MPM2 and PI and analyzed by flow cytometry to determine the percentage of cells in each phase of the cell cycle. PI staining determines the DNA content while the cells positive for MPM2 are the mitotic cells. As by entry to mitosis many proteins are phosphorylated either directly or indirectly by M-phase-promoting factor, MPM2 stains phosphorylated M-phase proteins, giving an indication about the number of cells on the M-phase of the cell cycle.

As shown in Fig. 3, HCT116 cells that either express HGyv-Apoptin or CAV-Apoptin show a G2/M arrest as compared to the cells that express the control vector. G2/M arrest occurs prior to apoptosis as upon HGyv-Apoptin expression; cells arrest at the G2 phase with a sub-G1 degraded DNA content cells undergoing apoptosis starting to accumulate.

HGyv-Apoptin Induces an Abnormal Spindle Formation

To further investigate the effect of HGyv-Apoptin on cell cycle, we have checked the effect of HGyv-Apoptin expression on HCT116 mitotic cells. Recent studies have shown that CAV-Apoptin induces abnormal spindle formation

in cancer cells leading to mitotic catastrophe (Lanz et al. 2013). To check whether HGyv-Apoptin has a similar effect, cells were stained with endogenous Tubulin to check bipolar spindles formation. Cells that express HGyv-Apoptin show an abnormal spindle as compared to the control cells. We have scored the number of cells expressing either of the vectors and showing an abnormal spindle. The results prove that HGyv-Apoptin induces an abnormal spindle formation in mitotic cells. HGyv-Apoptin mitotic expressing cells with abnormal spindle were greatly enlarged as compared to the control cells (Fig. 4). These data further support that HGyv-Apoptin has a similar mechanism of action as its homologue CAV-Apoptin.

HGyv-Apoptin Effects on Cell Cycle Regulators

To further understand HGyv-Apoptin mechanism of action, we have studied its effects on cell cycle regulators. The APC/C ubiquitination complex plays a major role in mitosis, with its cdc20 subunit critical for meta- to anaphase transition, and its CDK/Cyclin subunit regulating the mitotic exit. The ubiquitination of cyclin B allows for inactivation of CDK1/Cyclin B complex and the mitotic exit while CDK1/cyclin B1 complex prevents cell cycle exit (Malumbres and Barbacid 2005).

We thus evaluated the level of cyclin B1 expression in HCT116 cells that express either HGyv-Apoptin or CAV-Apoptin as compared to the cells that express the control empty GFP vector. HGyv-Apoptin as well as CAV-Apoptin expressing cells show a sustained high level of cyclin B1, as compared to the positive control HCT116 cells treated with the cell cycle arrest inducer Nocodazole, or the negative control HCT116 cells that were transfected with the control vector. In addition, normal primary human fibroblasts expressing HGyv-Apoptin show low level of cyclin B1 consistent with a normal progression of the cells through the cell cycle. These data suggest that HGyv-Apoptin interferes with the cell cycle most likely via inhibiting the APC function, thus blocking the exit from the cell cycle selectively in cancer cells while having no effect on cell cycle progression in normal cells (Fig. 5).

Discussion

Two viral PKTCs E4orF4 and CAV-Apoptin kill cancer cells by interference with the cell cycle, and by induction of G2/M arrest and apoptosis (Kornitzer et al. 2001; Lanz et al. 2013; Maddika et al. 2009). Earlier research from our group shows that CAV-apoptin presence in the cell leads to the activation of CDK2, and that the activated CDK2 is responsible for the phosphorylation of CAV-Apoptin at critical for nuclear retention, Thr-108 (Maddika

Fig. 3 HGyv-Apoptin induces G2/M arrest. **a** Cell cycle analysis using PI and MPM2 staining (indicator of phosphorylated M-phase proteins and the percentage of cells on the M-phase of the cell cycle). At the indicated time points, post-transfection, cells were stained overnight with anti-MPM-2 antibody, then washed three times the next morning, and stained with the anti-cy5 secondary antibody, followed by propidium iodide staining. While the cells that express the control vector progress normally through $>h$ out the cell cycle, cells that either express HGyv-Apoptin or CAV-Apoptin show a G2/M arrest. G2/M arrest occurs prior to apoptosis as upon HGyv-Apoptin or CAV-Apoptin expression; cells arrest at the G2 phase with an accumulation of sub-G1 degraded DNA content cells undergoing apoptosis. Green (FL-1, GFP-positive) cells were gated and analyzed

among the whole population of cells. FL3 corresponds to linear propidium iodide fluorescence measurement and FL4 corresponds to cyc 5 integrated fluorescence measurements. **b** Quantification of the percentage of cells at the G2 (black bars) or M phase (white bars) of the cell cycle, cells that either express HGyv-Apoptin or CAV-Apoptin show an arrest at the G2 phase of the cell cycle as compared to the cells that express the control vector. The mean of G2 arrested cells was calculated from three independent experiments. Both HGyv-Apoptin and CAV-Apoptin show significantly more G2-arrested cells than the control group ($P < 0.01$) ($P = 6.0300044652859E-11$ for HGyv-Apoptin cells and $3.6211569068185E-11$ for CAV-Apoptin cells)

et al. 2009). CAV-Apoptin also causes nuclear transfer of Akt kinase that then phosphorylates in the nucleus several proteins contributing to CAV-Apoptin's toxicity (Jain et al. 2016; Maddika et al. 2007). Our presented data here indicate that HGyv-Apoptin also follows the same toxicity mechanism to kill tumor cells (Fig. 6). All the viral PKTCs interfere with the APC/C function suggesting a common mechanism for the detection and the elimination of tumor cells undoubtedly associated with viral replication.

To further understand viral PKTCs behavior in tumor cells and their selective toxicity which is highly associated with their nuclear localization and accumulation, it would be important to understand the significance of the

nuclear accumulation of HGyv-Apoptin in cancer cells, and to identify its nuclear interacting partners. Studies have shown that CAV-Apoptin shuttles in both tumor and normal cells but only accumulates in the nuclei of tumor cells, we tempt to speculate that apoptins may sequester components essential for the cell cycle progression like APC1. For example, APC1 may be sequestered in promyelocytic leukemia bodies by CAV-Apoptin (Heilman et al. 2006). It would be interesting to check if HGyv-Apoptin shows the same cellular behavior, to identify other nuclear interacting partners and to understand to which extent this shuttling is essential for its apoptotic activity. In the proposed model, the inhibition of the nuclear export of HGyv-Apoptin

Fig. 4 HGyv-Apoptin induces an abnormal spindle. **a** Spindle formation in HGyv-Apoptin expressing HCT116 cells. Cells were fixed 48 h post-transfection and stained with DAPI for the detection of nuclear morphology and with anti-tubulin antibody for the detection of spindle formation. **b** Quantification of the percentage of cells with abnormal spindle and either expressing the control vector or HGyv-Apoptin. Per experiment, 100 mitotic cells that were positive for GFP-HGyv-Apoptin and pEGFPC1 were scored. HGyv-Apoptin-positive mitotic cells contained significantly more abnormal spindles ($P < 0.01$) than the control vector ($P = 2.2006711030126E-11$)

Fig. 5 HGyv-Apoptin effects on cell cycle regulators. **a** HGyv-Apoptin expression results in sustained levels of Cyclin B1 expression, showing that HGyv-Apoptin interferes with the APC/C ubiquitin function. The decline of cyclin B through degradation by APC/C is required for the completion and the exit from the cell cycle. **b** Densitometric representation of cyclinB1/beta-actin ratio from “A”

should abolish its toxicity. In this case, a decreased apoptosis and a decreased nuclear level of HGyv-Apoptin should be observed. To test the proposed model of HGyv-Apoptin action, one could employ HGyv-Apoptin mutants lacking ability to enter the nucleus, either by mutating critical elements of the bipartial nuclear localization signal, or by mutating the critical Threonine close to the C-terminus of the protein.

Viruses may not only be the source of proteins with selective anticancer properties, but also may contribute to carcinogenesis, like for example human papillomaviruses (cervical and oral cancer) (Liu et al. 2016; Zaravinos 2014), human T-cell leukemia virus (hairly cell leukemia) (Chlichlia et al. 2002; Los et al. 1998) etc. Like in some other diseases (Likus et al. 2016a), it is however sometimes difficult to clearly associate the given pathogen with cancer etiology. Many viruses preferentially or exclusively replicate in dividing cells; hence, their components are potentially becoming an important source/inspiration in the search of drugs that preferentially target cancer stem cells (Akbari-Birgani et al. 2016; Farahani et al. 2014; Wasik et al. 2014). Beside viral methods, some clinically tested as well as new drugs with preferential toxicity towards cancer stem cells are increasingly becoming available (Jangamreddy et al. 2013; Likus et al. 2016b; Moosavi et al. 2016). Such tasks are facilitated by recent progress in methodologies that allows for better detection and monitoring of cancer stem

Fig. 6 Proposed model for HGyv-Apoptin mechanism of action. In cancer cells, nuclear HGyv-apoptin induces abnormal spindle formation and serves most likely to the nuclear sequestration of components essential for the cell cycle progression like APC1. This leads to a weakened SAC and sustained high levels of cyclin B1 leading to G2/M arrest and apoptosis

cells (Cieslar-Pobuda et al. 2015, 2016; Likus et al. 2016c). While it is unlikely that a universal cancer cure will be found, rapid development will certainly lead to efficient anticancer strategies targeting groups of cancers.

Acknowledgements SG acknowledges Manitoba Medical Service Foundation, and University of Manitoba Research Grant Program. MJL kindly acknowledges the support from NCN Grant #: 2016/21/B/NZ1/02812.

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