INSTITUT PASTEUR

Microbes and Infection 11 (2009) 646–653

www.elsevier.com/locate/micinf

Original article

Host cell-specific effects of lentiviral accessory proteins on the eukaryotic cell cycle progression

Mari Matsuda, Azusa Arai, Yusuke Nakamura, Ryuichi Fujisawa, Michiaki Masuda*

Department of Microbiology, Dokkyo Medical University School of Medicine, Kita-kobayashi 880, Mibu, Tochigi 321-0293, Japan

Received 6 February 2009; accepted 17 March 2009
Available online 1 April 2009

Abstract

Lentiviral accessory proteins are thought to play important roles in regulating the viral replication through modulation of host cell functions. For example, Vpr of human immunodeficiency virus type 1 (HIV-1) induces the cell cycle G2 arrest in a host cell-specific manner. Similarly, HIV-2 Vpr, but not Vpx, has been shown to induce G2 arrest in primate cells. It has also been reported that Orf-A of feline immunodeficiency virus (FIV) induces G2 arrest in a simian cell line. However, activities of these non-HIV-1 accessory proteins in different cellular context are unclear. In this study, effects of HIV-2 Vpr, Vpx and FIV Orf-A on cell cycle progression were compared with those of HIV-1 Vpr in various mammalian cell lines and the fission yeast. These non-HIV-1 accessory proteins induced the cell cycle arrest in a host cell-specific manner, and their specificities were different from each other. Interestingly, HIV-2 Vpx-induced G2 arrest in bovine MDBK cells. It was also notable that HIV-2 Vpx and FIV Orf-A appeared to block the cell separation in the fission yeast. The host cell-specific activities of different lentiviral accessory proteins revealed in this study may provide a useful basis for elucidating the mechanism of their functions.

Keywords: Human immunodeficiency virus (HIV); Feline immunodeficiency virus (FIV); Accessory protein; Vpr; Vpx; Orf-A; Cell cycle; Fission yeast

1. Introduction

Viruses of genus *Lentiviridae* are complex retroviruses encoding regulatory and accessory proteins in addition to GAG, POL and ENV proteins common to all retroviruses. While regulatory proteins are universally indispensable for the viral replication in any cells, accessory proteins are not necessarily essential in certain cells. However, the accessory proteins are thought to play important roles in regulating the viral replication through modulation of host cell functions. Among the lentiviral accessory proteins, Vpr of human immunodeficiency virus type 1 (HIV-1) arrests the host cell cycle at G2 phase [1]. It has been shown that the promoter activity of HIV-1 LTR is the highest in G2 phase of the cell cycle, suggesting that Vpr-induced G2 arrest might enhance HIV-1 production by increasing viral RNA synthesis [2]. The ability of HIV-1 Vpr to cause G2 arrest is host cell-specific, and different mammalian cell lines showed differential susceptibility to Vpr-induced G2 arrest [3]. Interestingly, the fission yeast, *Schizosaccharomyces pombe*, was also shown to be susceptible to HIV-1 Vpr-mediated G2 arrest [4–6].

Other primate lentiviruses, such as HIV-2 and simian immunodeficiency virus (SIV) encode a pair of closely related accessory proteins, Vpr and Vpx. While the ability of Vpr to induce G2 arrest in primate cells appears to be conserved among these viruses [7–10], Vpx hasn't been shown to affect cell cycle progression so far. In addition, effects of HIV-2 Vpr and Vpx in non-primate cells are largely unknown.

Another lentivirus, feline immunodeficiency virus (FIV), which can cause progressive immune suppression in cats, encodes an accessory protein, Orf-A. Although Orf-A has been shown to cause G2 arrest in simian cells [11], its effects in feline and other eukaryotic cells haven't been reported yet.

In this study, in order to examine the host cell-specificity of the function of HIV-2 Vpr, Vpx and FIV Orf-A, their effects on the cell cycle progression were examined in various

* Corresponding author. Tel.: +81 282 87 2131; fax: +81 282 86 5616.
E-mail address: m-masuda@dokkyomed.ac.jp (M. Masuda).

1286-4579/$ - see front matter © 2009 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.micinf.2009.03.006

mammalian cell lines and *S. pombe*. The results demonstrate that like HIV-1 Vpr, these accessory proteins affect the cell cycle progression in host cell-specific manners. Our study also contribute novel findings that HIV-2 Vpx could cause G2 arrest in mammalian cells and that HIV-Vpx and FIV Orf-A could apparently block the cell separation of the fission yeast. These results may serve as a useful basis for elucidating how these accessory proteins fulfill their functions, as well as the mechanism of eukaryotic cell cycle regulation.

2. Materials and methods

2.1. Mammalian cell culture

HEK 293 cells were grown in Dulbecco's modified Eagles's medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 μg/ml streptomycin at 37 °C with 5% CO_2. HeLa, Vero, CRFK, MDBK and ST Iowa cells derived from human (*Homo sapiens*), African green monkey (*Cercopithecus aethiops*), cat (*Felis catus*), cattle (*Bos taurus*) and pig (*Sus scrofa*) were also grown under the same conditions.

2.2. Adenoviral vector preparation and infection

DNA fragments encoding HIV-1_{NL4-3} Vpr, HIV-2_{ROD} Vpr and Vpx, and FIV Orf-A were amplified by PCR from respective infectious DNA clones [12–14]. Each of the DNA fragments was subcloned in pCruz-His plasmid (Santa Cruz Biotechnology, CA, USA) so that the His tag could be added to the amino terminus of respective accessory protein. Then, adenoviral vectors expressing both N-terminally His-tagged accessory protein and enhanced green fluorescent protein (GFP) in an internal ribosomal entry sequence (IRES)-dependent manner were constructed by using an Adeno-X expression system (Clontech, CA, USA) and propagated on HEK 293 cells as described previously [3]. The obtained vector virus was concentrated by using a Virapur adenovirus purification kit (LLS, CA, USA). Titers of the prepared vector viruses measured on HEK 293 cells were in the range of 1.3–4.4 × 10^8 $TCID_{50}$/ml. For vector infection, target cells were incubated with a fresh medium containing the vector virus for 1 h. Then, the virus-containing medium was replaced by regular culture medium.

2.3. Flow cytometry

To determine vector transduction efficiency, cells harvested at 48 h post-infection were suspended in cold phosphate-buffered saline (PBS) containing 2% FBS, and proportion of GFP-positive cells was measured by a flow cytometer (FACS Caliber, Becton Dickinson, CA, USA). For cell cycle analysis, cells were suspended in PBS containing 0.2% Triton-X, and RNase A and propidium iodide (PI) were added to final concentrations of 1 mg/ml and 25 μg/ml, respectively. Cellular DNA contents were measured by flow cytometry, and proportions of 2N and 4N cells were determined by using ModFit LT program (Verity Software House, ME, USA).

2.4. Laser scanning cytometry (LSC)

Cells seeded in a chamber slide were infected with the adenoviral vectors and 48 h post-infection, fixed by cold methanol for 10 min. Then, the cells were incubated with PI staining solution (50 μg/ml PI and 0.1 mg/ml RNase A in PBS) for 30 min at 37 °C and mounted with a 1:1 mixture of PI staining solution and glycerol. Cell cycle phases of individual cells were determined by using a laser scanning cytometer (LSC2, Olympus, Tokyo, Japan) on the basis of their DNA content and the degree of chromatin condensation.

2.5. Western blot analysis

Cells were seeded at a density of 2 × 10^6 cells in a 10-cm culture dish and infected with the adenoviral vectors. Twenty-four hours later, the cells were lysed in 1 ml of lysis buffer containing 100 mM NaH_2PO_4, 10 mM Tris–HCl (pH 8.0), and 6 M guanidine hydrochloride. One-fifth of each lysate was set aside for western blot detection of tubulin as a control for normalization. From the remaining lysate, each of His-tagged accessory proteins was precipitated by using nickel agarose beads (Qiagen, MD, USA) and loaded to a 12% NuPAGE Bis-Tris gel (Invitrogen, CA, USA) for electrophoresis. The samples were then transferred to a polyvinylidene fluoride membrane and reacted with an anti-His monoclonal antibody (Santa Cruz Biotechnology, CA, USA). The signals were detected by the chemiluminescence method using a horseradish peroxidase-conjugated anti-mouse IgG antibody (Amersham Bioscience, UK).

2.6. Analysis using the fission yeast

2.6.1. Expression vectors and transformation

DNA fragments encoding the His-tagged accessory proteins were excised from pCruz-His subclones by restriction digestion and inserted into the downstream of *nmt1* promoter of pREP-1 plasmid [15]. The constructed plasmids were introduced into *S. pombe* (*h⁻ leu1*) by using a Fast Yeast transformation kit (Geno Technology, MO, USA). The transformed yeast cells were spread on minimal medium (MM) agar plates containing thiamine (10 μM) and incubated at 30 °C for 4–6 days, and successfully transformed clones were isolated.

2.6.2. Protein analysis of S. pombe

S. pombe cells in the mid-log growth phase were seeded in MM without thiamine at a density of 3 × 10^5 per ml and grown at 30 °C for 18 h with vigorous shaking. Then, the cellular protein samples were prepared in the lysis buffer containing 100 mM NaH_2PO_4, 10 mM Tris–HCl (pH 8.0) and 8 M urea. The samples were subjected to electrophoresis, and His-tagged proteins were detected by western blotting as described above.