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Fluorescently-labeled RNA packaging into HIV-1 particles: Direct examination of infectivity across central nervous system cell types

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HIV penetrates the central nervous system (CNS), and although it is clear that microglia and to a lesser extent astrocytes are infected, whether certain other cell types such as neurons are infected remains unclear. Here, we confirmed the finding that RNAs of both cellular and viral origins are present in native HIV-1 particles and exploited this phenomenon to directly examine HIV-1 infectivity of CNS cell types. Using in vitro transcribed mRNAs that were labeled with a fluorescent dye, we showed that these fluorescent mRNAs were packaged into HIV-1 particles by directly examining infected cells using fluorescence microscopy. Cells in culture infected with these labeled virions showed the fluorescent signals of mRNA labels by a distinct pattern of punctate, focal signals within the cells which was used to demonstrate that the CXCR4-tropic NL4-3 strain was able to enter microglia and to a lesser extent astrocytes, but not neurons. The strategy used in the present study may represent a novel approach of simplicity, robustness and reliability for versatile applications in HIV studies, such as the determination of infectivity across a broad range of cell types and within sub-populations of an individual cell type by direct visualization of viral entry into cells.

1. Introduction

Retroviruses, including human immunodeficiency virus (HIV), replicate through a DNA proviral intermediate which is integrated into the host cell genome. Transcription of this proviral DNA gives rise to a primary, full-length transcript that acts as genomic RNA for packaging into progeny virions. Retroviruses selectively package two copies of viral genomic RNA per viral particle, which is mediated by the viral structural protein Gag. In addition to the viral genomic RNA, diverse retroviruses have been found to contain RNA species of both cellular and viral origins (Muriaux et al., 2001).

HIV type-1 (HIV-1)-spliced RNAs have been readily detected within infectious, native HIV-1 particles and these transcripts can be reverse-transcribed in both cells and virions (Houzet et al., 2007a,b; Liang et al., 2004). Many cellular RNAs of high abundance such as Y RNAs, 7SK and 7SL RNAs, 5S rRNA, U6 snRNA and GAPDH and β-actin mRNAs have also been found in HIV-1 particles (Tian et al., 2007). These results support the notion that HIV-1 packages various viral and cellular RNAs beyond the genomic viral RNA. There is also evidence that some RNA species such as 7SL and U6 RNAs are packaged into HIV-1 particles at a much higher efficiency than others (Didierlaurent et al., 2011; Tian et al., 2007), which implies the virus uses both selective and passive RNA packaging mechanisms.

It is well known that HIV-1 preferentially infects cells of the human immune system such as $CD4^+$ T cells, macrophages and dendritic cells. However, HIV infection causes diverse neurologic disorders including sensory neuropathy, myelopathy and HIV dementia and cognitive/motor disorder which are getting worldwide attention in HIV patients, collectively termed neuroAIDS (Power et al., 2009). Nonetheless, our understanding on HIV-1 infectivity of particular central nervous system (CNS) cell types has remained elusive. Due to the limited availability of primary human CNS cells, the high degree of phenotypic diversity within individual CNS cell types and the difficulty of maintaining them in culture, the current knowledge on HIV-1 infectivity of CNS cell types has been largely derived using indirect methods for examining the post-mortem brain tissue of HIV patients such as immunohistochemistry. Therefore, the development of methods to

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directly examine HIV-1 infectivity of limited numbers of individual cell types in culture may provide results that are more clear than those from techniques used to analyze whole human brain tissue. Microglia represent the resident macrophages of the brain and spinal cord, and are thus believed to serve as the principal target of HIV-1 infection in the CNS. Immunohistochemical examination of brain tissue from HIV patients supports that HIV-1 also infects astrocytes, although this cell type is infected at a much lower frequency than microglia and infection of astrocytes may depend more on the cellular environmental conditions (Carroll-Anzinger and Al-Harthi, 2006; Churchill et al., 2006, 2009; Gorry et al., 2003; Li et al., 2011; Messam and Major, 2000). However, it remains controversial whether HIV-1 infects neurons, although it is generally believed that neurons are not infected (reviewed in Kramer-Hammerle et al., 2005; Verma et al., 2010).

HIV entry into the cell is the beginning of the infectious process. In the present study, we first confirmed the diverse RNA packaging phenomenon of HIV-1, and demonstrated that fluorescently-labeled mRNAs from various species were able to be packaged into HIV-1 particles when used to directly examine infected HeLa MAGI-CCR5 cells by fluorescence microscopy. We then employed our strategy to examine HIV-1 infectivity across different primary human CNS cell types in culture by direct visualization of viral entry into these cells. We were able to readily observe fluorescent signals in microglia and astrocytes, but not neurons. We suggest that fluorescently-labeled RNA packaging by HIV-1 for direct visualization of viral entry represents a novel and simple approach to reliably determine infectivity across various cell types and within sub-populations of an individual cell type. Moreover, this method might be further developed to extend to other applications.

2. Materials and methods

2.1. Plasmids, cells and viruses

CXCR4-tropic HIV-1 NL4-3 proviral DNA plasmid was kindly provided by Dr. Fatah Kashanchi (George Mason University; Manassas, VA, USA). HeLa MAGI-CCR5 cells were obtained through the NIH AIDS Research and Reference Reagent Program (catalog 3522), Division of AIDS, NIAID: MAGI-CCR5 from Dr. Julie Overbaugh (Chackerian et al., 1997). Primary human microglia (catalog 1900-f1), astrocytes (catalog 1800) and neurons (catalog 1520) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured according to the manufacturer's instructions. HIV-1 strain IIIB (HIV-1_{IIIB}) was purchased from Advanced Biotechnologies (Columbia, MD, USA) where the virus was propagated in H9 cells and prepared as: (1) direct pelleted virus (catalog 10-124-000) concentrated from cell culture supernatant by centrifugation without further purification, or (2) purified virus (catalog 10-118-100) clarified from culture supernatant that was concentrated and cleaned of debris by ultracentrifugation and extraction of the viral band from a sucrose density gradient; both of these preparations contain infectious virus.

2.2. RT-PCR

Total RNA was isolated using the miRNeasy Mini Kit (Qiagen; Valencia, CA, USA). Following DNase treatment of RNA, reverse transcription was conducted using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Foster City, CA, USA). RT-PCR reactions were performed in a total volume of 20 μL containing SensiMix™ SYBR qPCR reagents (Bioline USA; Tauton, MA, USA) using a Corbett Rotor-Gene 6000 real-time PCR system (Qiagen). Sequences of the primer sets used for RT-PCR are listed in Table 1, and these primers were designed to span introns whenever possible. For detection of packaged RNAs in native HIV-1 particles (Fig. 1), RT-PCR conditions consisted of an initial hold step at 95 °C for 10 min, followed by 5 cycles (95 °C for 5 s, 62 °C/−1 °C each cycle for 10 s, 72 °C for 20 s) and 25 cycles (95 °C for 5 s, 58 °C for 10 s, 72 °C for 20 s). For detection of HIV-1 genomic RNA in supernatants from HEK-293T cells transfected with proviral DNA plasmid with and without fluorescently-labeled mRNAs (Fig. 2B), RT-PCR conditions consisted of an initial hold step at 95 °C for 10 min, followed by 5 cycles (95 °C for 5 s, 62 °C/−1 °C each cycle for 10 s, 72 °C for 20 s) and 35 cycles (95 °C for 5 s, 58 °C for 10 s, 72 °C for 20 s). The specificity of the amplified products was determined by melting curve analysis followed by agarose gel electrophoresis and sequencing. The agarose gels were stained with ethidium bromide, and images were taken using a Kodak Image Station 440 (Kodak; Rochester, NY, USA).

2.3. In vitro transcription and labeling of mRNA

The full-length coding cDNA fragment (261 bp) of HIV-1_{IIIB} Tat_{1-86} mRNA and the C-terminal coding cDNA fragment (432 bp) of mouse EndoG mRNA were prepared by PCR with the primers indicated in Table 1, while the C-terminal coding cDNA fragment (423 bp) of firefly luciferase mRNA was provided with the pGEM-T-easy vector system (Promega; Madison, WI, USA); these cDNA fragments were cloned into the pGEM-T-easy vector. The insert and direction in the resulting plasmids were confirmed by sequencing, and plasmid DNA was prepared using the Hipure Plasmid Filter Midiprep Kit (Invitrogen; Grand Island, NY, USA). Plasmids were linearized by restriction enzyme digestion, and in vitro transcription was performed to produce sense messenger RNA followed by covalently conjugating the Alexa Fluor® 594 fluorescent dye to the mRNAs using the FISH Tag™ RNA Red Kit (Invitrogen; catalog F32954) according to the manufacturer's instructions.

2.4. Packaging of fluorescently-labeled RNA into HIV-1 particles

HEK-293T cells (GenHunter; Nashville, TN, USA; catalog Q401) were co-transfected with HIV-1 NL4-3 proviral DNA plasmid and each fluorescently-labeled mRNA at a ratio of 1 μg to 0.6 μg, respectively, corresponding to molar ratios of 1:65 (Tat), 1:39 (EndoG) and 1:40 (luciferase), using Lipofectamine 2000 reagent (Invitrogen). After 48 h, the cell culture supernatants containing virus were collected, subjected to 1 freeze-thaw cycle and centrifuged at 13,200 r.p.m. for 5 min to remove cells and debris. Virus-containing supernatants were stored in aliquots at −80 °C.

2.5. HIV-1 treatments and fluorescence microscopy

Cells were cultured in 4-well glass chamber slides coated with poly-L-lysine. After overnight culture, cells were incubated with fluorescent mRNA-labeled HIV-1 for 24 h at 37 °C (24 h was chosen as the time point to ensure that all cell types had an ample opportunity to infect). Cells were then fixed in 3.7% paraformaldehyde/PBS for 15 min. After additional washing in PBS, coverslips were mounted onto the chamber slides using ProLong® Gold antifade reagent containing DAPI (Invitrogen). Samples were imaged using a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss; Thornwood, NY, USA), and images were edited with ImageJ (NIH; Bethesda, MD, USA) and Adobe Photoshop (Adobe Systems Incorporated; San Jose, CA, USA) software. For a control experiment (Fig. 2D), fluorescently-labeled Tat mRNA alone was added to cells grown in 4-well glass chamber slides at an amount of 0.1 μg per well and incubated for 24 h at 37 °C, followed by fixation and image recording using a Zeiss Axio Observor Z1 microscope system (Carl Zeiss). For co-detection of labeled mRNA in HIV-1-infected cells (Fig. 3), treated cells were fixed with 3.7% paraformaldehyde/PBS