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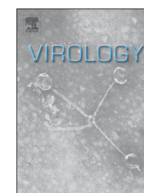

HIV-1 nucleocapsid protein localizes efficiently to the nucleus and nucleolus

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ABSTRACT

The HIV-1 nucleocapsid (NC) is an essential viral protein containing two highly conserved retroviral-type zinc finger (ZF) motifs, which functions in multiple stages of the HIV-1 life cycle. Although a number of functions for NC either in its mature form or as a domain of Gag have been revealed, little is known about the intracellular localization of NC and, moreover, its role in Gag protein trafficking.

Here, we have investigated various forms of HIV-1 NC protein for its cellular localization and found that the NC has a strong nuclear and nucleolar localization activity. The linker region, composed of a stretch of basic amino acids between the two ZF motifs, was necessary and sufficient for the activity.

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1. Introduction

HIV-1 nucleocapsid (NC) protein is derived from the viral Gag polyprotein precursor and is a small, basic nucleic acid binding protein containing two retroviral-type zinc finger (ZF) motifs. The NC exists in two different forms and functions in various stages of the HIV-1 life cycle. In the early phase of virus infection, it exists in the virion core as a fully processed mature protein (Briggs et al., 2004) and is involved in multiple reverse transcription steps (Chan et al., 1999; Tisne et al., 2001; You and McHenry, 1994; Tisne et al., 2004; Bampi et al., 2004; Levin et al., 2005). In the late phase of infection, from virus gene expression to assembly and budding, NC is a part of the Gag and Gag-Pol proteins, which specifically recognizes the packaging signal sequence (Ψ) in the full length vRNA genome and encapsidates it into a virus particle (Gorelick et al., 1993; Berkowitz et al., 1995; Poole et al., 2005). These activities are attributed to the nucleic acid binding and chaperone functions of NC. During virus budding and maturation, Gag protein is cleaved into individual proteins including NC into its mature form by the viral protease, which can bind and stabilize the viral genomic RNA (gRNA) in the virion, leading to maturation of infectious virus.

Although many functions of NC in both its mature form and as a domain of Gag are known, little is known about how intracellular localization of NC occurs or about its role in Gag protein trafficking. It was recently reported that Rous Sarcoma Virus (RSV) Gag is targeted to the nucleolus by a nucleolar localization signal in its NC domain, which was proposed to be similar to the HIV-1 NC (Lochmann et al., 2013). In HIV-1, however, matrix (MA) protein which is also derived from Gag has previously been shown to have a strong nuclear importing activity (Bukrinsky et al., 1993; Haffar et al., 2000), although this has recently been challenged (Baluyot et al., 2012; Depienne et al., 2000; Fouchier et al., 1997). Furthermore, the localization of HIV-1 Gag into the nucleus remains controversial. It was previously shown that HIV-1 Gag was excluded from the nucleus, unlike Feline Immunodeficiency Virus (Kemler et al., 2012) or RSV (Baluyot et al., 2012; Grewe et al., 2012), suggesting that nuclear localization and trafficking is not the same in all retroviral Gag proteins. However, detection of HIV-1 Gag in the nucleus has been reported in recent studies, although the region responsible for nuclear localization was not clearly defined (Grewe et al., 2012; Hermida-Matsumoto and Resh, 2000), supplement data of Lehmann et al. (2009)).

Previously, mature HIV-1 NC was detected migrating from the cytoplasm to the nucleus and accumulating in the nucleus at 8 h post-infection (Zhang and Crumpacker, 2002; Gallay et al., 1995). However, because these observations were made in a context of using whole virus, it still remains to be determined whether NC itself is able to target the nucleus independently. A recent report, using YFP fusions of HIV-1 NC and mutants, demonstrated that NC could localize to the nucleolus and two regions were reported to

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be necessary, aa 10-11 and aa 32-34 (Lochmann et al., 2013). To date, however, the subcellular location and nuclear localization activity of mature unfused wild-type HIV-1 NC remains to be determined, and has been deferred partly due to the absence of an expression vector system capable of expressing the NC to a high level.

Thus, we have undertaken the present study to understand further the subcellular localization of the HIV-1 NC. The results show that NC has strong nuclear and nucleolar localization activity and thus might be an important factor in nucleus entry of the HIV-1 preintegration complex.

2. Materials and methods

2.1. Plasmid construction

For the expression of NC and NC zinc finger mutants, the Gag/Pol region of pLP1 (Invitrogen, CA) was deleted by digestion with *Pml*I and *BspE*I and replaced with codon-optimized NC or zinc finger NC mutant fragments, which were chemically synthesized (GenScript, NJ). For the EGFP fusion NC series, each NC or NC mutant was amplified by PCR, forward-flanked with an *Nde*I site and reverse-flanked with a *BspE*I site with the stop codon deleted. To create the $NC_{Lin\text{-}Ala}$ and $NC_{F16AW37A}$ mutants, we used an overlap extension PCR method (Ho et al., 1989). For $NC_{F16AW37A}$, we first created NC_{F16A} using overlap PCR, which was then used as a template for $NC_{F16AW37A}$. EGFP was also amplified by PCR, flanked with *BspE*I and *Pst*I sites. NC or NC mutants and EGFP PCR products were then digested with *BspE*I and ligated together. The ligation product was amplified using the NC or mutant NC forward primer with the EGFP reverse primer, and the PCR product was blunted using Klenow DNA polymerase (New England Biolabs, UK), digested with *Pst*I and then inserted into *Pml*I and *Pst*I-digested pLP1 (a *Pst*I site was first inserted into pLP1, upstream of the *BspE*I site). Various Gag deletion mutants were also generated by PCR. The forward primers were forward-flanked with a *Pml*I site (a *Sma*I site in the case of CA) and reverse- flanked with a *BspE*I site. All primers used in this study are listed in the Supplementary Table 1.

2.2. Cell culture

HeLa and 293T cells were maintained in Dulbecco's modified Eagle medium (Pierce Hyclone, CA) and MT-4 cells were maintained in RPMI-1640 medium (Pierce Hyclone, CA), both supplemented with 10% foetal bovine serum (Pierce Hyclone, CA) and penicillin and streptomycin (Invitrogen, CA), and incubated at 37 °C in 5% CO_2.

2.3. Immunofluorescence (IF) analysis

IF was performed as described previously (Hameau et al., 2001). Briefly, HeLa cells were seeded on coverslips (Marienfeld, Germany) at 2.5×10^4 cells/well in 12-well tissue culture plates. The next day, cells were transfected with the indicated plasmids, using jetPEI according to the manufacturer's protocol (Polyplus-transfection, NY). After 24 h, the cells were washed with 1 × PBS and fixed in 4% paraformaldehyde. The fixed cells were permeabilized for 20 min, blocked in 0.1% Triton X-100, 2% BSA, and 5% normal horse serum for 30 min, and then incubated with the indicated antibody for 2 h. Cells were washed three times with 1 × PBS/0.1% Tween-20 (1 × PBST) for 10 min each and incubated with fluorescent-conjugated secondary antibody in the dark for 1 h, followed by two washes in 1 × PBST for 10 min each. Cells were counterstained with DAPI (1 μg/ml in 1 × PBST) for 10 min to detect nuclei. Coverslips were mounted onto glass sides with a 10 μl drop of Vectashield h-1000 solution (Vector Laboratories Inc., CA) and sealed with nail polish. All steps were performed at room temperature.

2.4. Subcellular fractionation and western blotting

HeLa cells (5×10^5 or 7×10^5 293T cells/well) were seeded in 6-well tissue culture plates and transiently transfected with the indicated plasmids using jetPEI. Twenty hours later, 20 nM of Leptomycin B (LMB) and 10ug/ml of cycloheximide (CHX) were treated. Four hours later, cells were washed with 1 × PBS and separated into two aliquots. For analysis of whole lysates, 1/10 volume of cells were lysed in 50 μl of RIPA buffer supplemented with protease inhibitors (2 mM PMSF (phenylmethylsulfonyl fluoride) and 1 μl protease inhibitor cocktail per 100 μl (P8340, Sigma, USA)). For fractionation studies, 9/10 volumes of cells were resuspended in 300 μl of buffer A (10 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 DTT, and 0.05% NP40, supplemented with protease inhibitors) and incubated for 10 min on ice. The cells were centrifuged at 1000 g for 10 min at 4 °C. The supernatant containing the cytoplasmic extract was transferred to a new 1.5 ml tube and centrifuged again (13,000 rpm for 3 min at 4 °C) to remove any remaining nuclear contamination. The pellet was washed in cold 1 × PBS to remove any remaining cytoplasmic contamination and then lysed in 150 μl of nuclear extraction buffer (20 mM HEPES, pH 7.5, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 25% glycerol, supplemented with protease inhibitors). The protein concentrations were determined by the Bradford method (BioRad, USA) and 5-15 μg of protein was subjected to Western bolt analysis.

2.5. Antibodies

Primary antibodies used in this study include the following: rabbit polyclonal anti-NC (custom antibody, abfrontier, Korea); mouse monoclonal anti-p24 (ab 9071, Abcam, MA); mouse monoclonal anti-B23 (sc-56622, Santa Cruz, CA); mouse monoclonal anti-LMNA (sc-56139, Santa Cruz, CA); goat polyclonal anti-TIAR (sc-1749, Santa Cruz, CA); mouse monoclonal anti-Calnexin (sc-70841, Santa Cruz, CA); mouse monoclonal anti-MTCO2 (ab 3298, Abcam, MA). Secondary antibodies included: Immunopure Goat anti-Mouse IgG (H+L) HRP conjugate (31430, Thermo Scientific, USA) and Immunopure Goat anti-Rabbit IgG (H+L) HRP conjugate (31460, Thermo Scientific, USA).

3. Results

3.1. NC localizes in the nucleus, nucleolus and cytoplasm

To examine the subcellular localization of HIV-1 NC, we transfected HeLa cells with a plasmid expressing the mature form of NC protein in a high level and found that NC localizes throughout the entire cell, including in the nucleus and nucleolus (Fig. 1A). To confirm the nuclear localization ability of NC and exclude any cell-specific effects or problems with immunofluorescence, including non-specific or false-positive staining, we undertook further the following approaches: (1) examining cytoplasmic and nuclear fractionates of 293T cells transfected with the same plasmid and (2) probing the localization of an EGFP-fused NC (NC-EGFP) in HeLa and MT-4 (human CD4 T cell line) cells. As observed in the immunofluorescence study,

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