European Journal of Cell Biology 90 (2011) 913–921

Contents lists available at ScienceDirect

European Journal of Cell Biology

journal homepage: www.elsevier.de/ejcb

Nef does not inhibit F-actin remodelling and HIV-1 cell–cell transmission at the T lymphocyte virological synapse

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ARTICLE INFO

Article history:
Received 29 July 2010
Received in revised form 7 September 2010
Accepted 22 September 2010

Keywords:
HIV-1 cell–cell spread
Nef
Virological synapse
T lymphocyte
Virion infectivity
F-actin polarization and remodelling

ABSTRACT

Nef, a HIV-1 pathogenesis factor, elevates virus replication *in vivo* and thus progression to AIDS by incompletely defined mechanisms. As one of its biological properties, Nef enhances the infectivity of cell-free HIV-1 particles in single round infections, however it fails to provide a significant and amplifying growth advantage for HIV-1 on such virus producing cells. A major difference between HIV-1 cell-free single round infections and virus replication kinetics on T lymphocytes consists in the predominant role of cell-associated virus transmission rather than cell-free infection during multiple round virus replication. HIV-1 cell-to-cell transmission occurs across close cell contacts also referred to as virological synapse (VS) and involves polarization of the F-actin cytoskeleton, formation of F-actin rich membrane bridges as well as virus budding to cell–cell contacts. Since Nef potently interferes with triggered actin remodelling in several cell systems to reduce e.g. cell motility and signal transduction, we set out here to address whether Nef also affects organization and possibly function of the T lymphocyte VS. We find that in addition to increasing infectivity of cell-free virions, Nef can also moderately enhance single rounds of HIV-1 cell–cell transmission between Jurkat T lymphocytes. This occurs without affecting cell conjugation efficiencies or polarization of F-actin and HIV-1 p24Gag at the VS, identifying actin remodelling at the VS as an example of Nef-insensitive host cell actin rearrangements. However, Nef-mediated enhancement of single round cell-free infection or cell-to-cell transmission does not potentiate over multiple rounds of infection. These results suggest that Nef affects cell-free and cell-associated HIV-1 infection by the same mechanism acting on the intrinsic infectivity of HIV-1 particles. They further indicate that the high efficacy of cell-to-cell transmission can compensate such infectivity defects. Nef therefore selectively interferes with actin remodelling processes involved in antiviral host cell defense while actin driven processes that promote virus propagation remain unaltered.

Introduction

The accessory protein Nef of primate lentiviruses is a key determinant for disease progression *in vivo* that elevates virus titers over several magnitudes during the first weeks of infection (Deacon et al., 1995; Hanna et al., 1998; Kestler et al., 1991). HIV-1 Nef hijacks a large variety of host cell membrane transport and signal transduction processes (Geyer et al., 2001; Kirchhoff et al., 2008; Roeth and Collins, 2006), the underlying molecular mechanisms as well as their relative contribution to virus spread *in vivo* however remain to be fully defined. A major limitation to our understanding how Nef enhances virus replication in infected individuals consists of the lack of pronounced effects of Nef on HIV-1 spread in most *ex vivo* cell culture systems. On the other hand, Nef is well-known to enhance virion infectivity in a single round of infection via a mechanism that is exerted in the virus producing cell but is manifested at the early post entry steps during target cell infection (Aiken and Trono, 1995; Chowers et al., 1994; Schwartz et al., 1995). The molecular mechanism of this activity is still unclear but has been suggested to require the association of Nef with the host cell GTPase dynamin (Pizzato et al., 2007). Incorporation of Nef into virus particles as well as the modulation of the virion lipid composition is not sufficient for the enhanced infectivity of HIV-1 particles (Brugger et al., 2007; Laguette et al., 2009). Although both parameters have not been directly correlated in a single experiment, Nef does not provide HIV-1 with a significant growth advantage in most cell lines even though they are known to support Nef's enhancing effect on virion infectivity. In line with this observation, Nef's ability to elevate single round virion infectivity and multiple round virus spread could be genetically uncoupled, even though both activities are conserved among lentiviral Nef proteins (Munch et al., 2007). While enhancement of virion infectivity has thus become a highly robust and widely accepted biological activity of Nef, its mechanism as well as relevance to HIV-1 spread *in vitro* and *in vivo* is unclear.

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0171-9335/$ – see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.ejcb.2010.09.010

In addition to infection with cell-free virions, the importance of cell-associated spread across connecting membrane bridges and close cell–cell contacts referred to as virological synapse (VS) for HIV-1 propagation is increasingly recognized and meanwhile thought to constitute the predominant mechanism of HIV-1 propagation in T lymphocyte cultures (Blanco et al., 2004; Haller and Fackler, 2008; Jolly et al., 2004; Mothes et al., 2010; Rudnicka et al., 2009; Ruggiero et al., 2008; Sato et al., 1992; Sattentau, 2008; Sherer et al., 2007; Sherer and Mothes, 2008; Sol-Foulon et al., 2007; Sourisseau et al., 2007; Sowinski et al., 2008). HIV-1 transmission across the VS depends on cell polarization including dynamic reorganization of the actin cytoskeleton and recruitment of virion components to cell–cell contacts (Hubner et al., 2009; Jolly et al., 2007a,b). Nef potently affects T lymphocyte morphology and actin remodelling to interfere with chemotaxis as well as with signal transmission at the immunological synapse (IS) presumably to prevent lymph node homing and hyperactivation of infected T lymphocytes (Arhel et al., 2009; Fackler et al., 2007; Haller et al., 2006; Nobile et al., 2009; Stolp et al., 2009, 2010; Thoulouze et al., 2006). IS refers to close contacts between antigen presenting cells and T lymphocytes that are closely related in organization and polarization to the VS (Piguet and Sattentau, 2004). Whether in analogy to its activities at the IS Nef also affects the architecture of the T lymphocyte VS to modulate HIV-1 cell–cell spread has not yet been addressed. The aims of this study were therefore to compare effects of Nef on cell-free infection and cell–cell HIV-1 transmission in T lymphocytes, to investigate their role for HIV-1 spread over multiple rounds of infection and to address morphological consequences of Nef expression at the VS.

Materials and methods

Cells and reagents

Jurkat E6.1 cells were cultivated in RPMI 1640+GlutaMAX™-1 supplemented with 10% fetal calf serum and 1% penicillin–streptomycin (all from Invitrogen). The reporter cell line E6.LTR1.GFP was generated by introducing a NL4-3 LTR-GFP reporter sequence (Michel et al., 2009) into E6.1 cells via electroporation. Stably transfected cells were selected and kept in the presence of Geneticin (Gibco) and sorted for constitutive low GFP expression (FACSAria™ cell sorter, BD). The following reagents were used: CellTrace dye CFSE Far red (Molecular Probes); the HIV-1 fusion inhibitor T20 (enfuvirtide, Fuzeon) (Roche); the reverse transcriptase inhibitor Sustiva (efavirenz) (Bristol-Meyers Squibb); transwell inserts with 3 μm pore size (Corning); anti-p24CA antibody (KC57-FITC) for intracellular staining and immunofluorescence analyses (Coulter), rabbit anti-CA antibody (Muller et al., 2004), FITC-conjugated anti-rabbit secondary antibody Alexa Fluor-488 (Invitrogen), TRITC-conjugated Phalloidin, Poly-L-lysine and fibronectin (all from Sigma), Aqua-Poly/Mount mounting medium (Polysciences).

HIV-1 production, cell-free infection and relative infectivity

Virus stocks of HIV-1_{NL4-3} expressing Nef from HIV-1_{SF2} (wt) or its *nef*-deleted counterpart (ΔNef) were generated by transfection of proviral HIV plasmids into 293T cells as described (Fackler and Krausslich, 2006). The HIV-1 p24CA antigen concentration was determined by p24 antigen enzyme-linked immunosorbent assay (ELISA) of the cell supernatant as described (Fackler and Krausslich, 2006).

For single round replication analyses of parental E6.1 and E6.LTR1.GFP cells, 2×10^6 cells were infected with 2000 ng p24CA of the 293T-derived cell-free viral stocks. After 4 h, cells were washed to remove input virus and incubated for further 44 h. Cells were fixed in PBS/2% PFA for 1.5 h and productive infection was determined by flow cytometry (FACSCalibur with CellQuest Pro 4.0.2 Software, BD Pharmingen) measured by GFP expression (E6.LTR1.GFP cells) and intracellular anti-p24CA stain (KC57-FITC 1:100 in PBS/0.1% Triton for 30 min) (parental E6.1 cells), respectively (Keppler et al., 2006).

For multiple round infection analyses on E6.1 and E6.LTR1.GFP cells, 2×10^6 cells were infected with 50 ng p24CA viral input which was washed out after 4 h. At several time points p.i., supernatant was removed, replaced by fresh medium and investigated for p24CA production by ELISA. In parallel, culture supernatants were analyzed for their relative infectivity in a standard TZM-bl reporter cell assay as described (Keppler et al., 2005).

Cell–cell transmission assay

To follow cell-associated transfer of HIV-1, a co-culture assay was adopted (Sourisseau et al., 2007). As donor cells, cultures of E6.1 cells were kept continuously infected (30–60% HIV-1 positive cells) with HIV-1 wt or ΔNef by regularly mixing in a high ratio (up to 1:100) with uninfected fresh cells. 1×10^6 E6.LTR1.GFP reporter cells, labelled with the CellTrace dye CFSE Far red (15 min at 37 °C), were used as target cells and mixed with an equal number of infected E6.1 donor cells (each population in 250 μl medium). Viral transfer was allowed to occur for 3–4 h and was stopped by the addition of the fusion inhibitor T20 (100 μM). Cells were incubated for various time points, then washed in PBS, fixed with PBS/2% PFA for 1.5 h and analyzed by flow cytometry. The relative amount of GFP expressing target cells (dye/GFP double positive cells relative to all dye positive cells) was calculated as a measure of transmission efficiency. Analogously, the same procedure was applied using parental E6.1 cells as targets. In this case however, after fixation, cells were stained for intracellular p24CA. Each experiment included several controls: The contribution of cell-free infection was determined by separating donor and target cell population by a transwell insert (Trw). As specificity control, a co-culture sample was directly fixed after mixing at time point zero (0 h). Furthermore, separate pre-incubation of donor and target cells with T20 (100 μM for 1 h) (T20) or Sustiva (10 μM for 1 h) (Sus) was performed to block productive infection by any route of transmission. Co-culture assays for wt and ΔNef HIV-1 transmission were always carried out in parallel. Normalization to identical numbers of infected input donor cells was based on prior determination of the percentage of productively infected cells by intracellular p24 staining and FACS analysis. For kinetic analyses, the overall volume of mixing reactions was increased keeping the cell density at 4×10^6/ml and cell aliquots were removed and analyzed at different time points. In order to compare different donor to target cell ratios, donor and target cell populations were mixed at a final concentration of 4×10^6 cells/ml using varying amounts of donor and target cells.

For detection of multiple cell–cell transmission events, the ratio of infected to uninfected cells was typically adjusted to 5:95. Every 24 h, half of the cells were removed and replaced by fresh uninfected E6.LTR1.GFP target cells. Harvested target cells were analyzed for infection levels as determined by GFP expression by flow cytometry. In parallel, the amount of released p24CA into the supernatant was measured by ELISA.

Immunofluorescence analyses of cell conjugates and membrane bridges

Formation and architecture of conjugates between T lymphocytes were analyzed by mixing productively HIV-1 and ΔNef infected E6.1 donor cell populations with uninfected E6.1 target