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A high throughput Cre–lox activated viral membrane fusion assay identifies pharmacological inhibitors of HIV entry

Anthony M. Esposito [a], Pamela Cheung [b], Talia H. Swartz [a], Hongru Li [a], Tshidi Tsibane [c], Natasha D. Durham [a], Christopher F. Basler [c], Dan P. Felsenfeld [b,1], Benjamin K. Chen [a,*]

[a] Division of Infectious Diseases, Department of Medicine, Icahn School of Medicine at Mount Sinai, Immunology Institute, New York, NY, United States
[b] Integrated Screening Core, Icahn School of Medicine at Mount Sinai, New York, NY, United States
[c] Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY, United States

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ABSTRACT

Enveloped virus entry occurs when viral and cellular membranes fuse releasing particle contents into the target cell. Human immunodeficiency virus (HIV) entry occurs by cell-free virus or virus transferred between infected and uninfected cells through structures called virological synapses. We developed a high-throughput cell-based assay to identify small molecule inhibitors of cell-free or virological synapse-mediated entry. An HIV clone carrying Cre recombinase as a Gag-internal gene fusion releases active Cre into cells upon viral entry activating a recombinatorial gene switch changing dsRed to GFP-expression. A screen of a 1998 known-biological profile small molecule library identified pharmacological HIV entry inhibitors that block both cell-free and cell-to-cell infection. Many top hits were noted as HIV inhibitors in prior studies, but not previously recognized as entry antagonists. Modest therapeutic indices for simvastatin and nigericin were observed in confirmatory HIV infection assays. This robust assay is adaptable to study HIV and heterologous viral pseudotypes.

Introduction

In order to enter the host cell, enveloped HIV particles must undergo membrane fusion with the host cell membrane. Many assays have been developed to interrogate this process in vitro, which can be categorized in two broad categories. The first category is cell-based fusion assays, in which viral envelope proteins are expressed on the surface of cells which are co-cultured with receptor-expressing cells; syncytia formation between the two cell types is detected via activation of a reporter gene expression (Herschhorn et al., 2011; Huerta et al., 2002; Sakamoto et al., 2003). These assays may not fully recapitulate the unique composition of lipids and proteins found on virus particles. The second category of fusion assay is virus particle based, where lipid membrane probes act as sensors of membrane mixing (Lowy et al., 1990; Raviv et al., 2002) or viral contents release into a target cell serve as measures of viral entry. A practical example of this type of assay utilizes the beta-lactamase (Blam) reporter gene fused to HIV Vpr, which is efficiently packaged into virus particles and then delivered into target cells upon virus fusion (Cavrois et al., 2002). Fusion activity is measured by cleavage of the fluorescent reporter substrate CCF2-AM that is loaded into the target cell cytoplasm, which causes in a shift in its fluorescent emission. This assay can provide a reliable indicator for HIV entry, however it is not ideal for high throughput screens due to the high cost of the CCF2-AM substrate and a complex assay protocol that requires numerous wash and incubation steps.

Viral membrane fusion assays have primarily been examined in the context of cell-free HIV entry. HIV particles infect $CD4^+$ T cells after exposure to cell-free virions, yet studies have revealed that infections mediated by cell–cell contact can greatly enhance the efficiency of viral dissemination in vitro (Dimitrov et al., 1993; Sourisseau et al., 2007). An adhesive junction between infected and uninfected T cells has been called the virological synapse (VS) and is defined by the localization of viral proteins Gag and Env and cellular receptor, CD4 to the site of cell–cell contact (Jolly et al., 2007). Live confocal imaging of HIV virological synapses revealed that the viral Gag protein was dynamically recruited to the site of cell–cell adhesion where newly formed viruses are translocated into the target cell through an endocytic process (Hubner et al., 2007). The transfer of virus from cell-to-cell requires active targeting of viral proteins to the VS in the donor cell, and the endocytic internalization process also appeared to require active signaling. Cellular inhibitors such as actin antagonists or certain antibodies have differential abilities to inhibit HIV infection via VS

* Corresponding author.
E-mail address: benjamin.chen@mssm.edu (B.K. Chen).
[1] Current address: CHDI Management/CHDI Foundation, Princeton, NJ, United States.

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and cell-free virus (Durham et al., 2012). Recent studies have revealed that the high copy number of incoming virus during VS require higher concentrations of reverse transcriptase inhibitors to block and thus may contribute to reservoirs (Sigal et al., 2011). Because of the aspects of viral entry that may be unique to virological synapses, we set out to develop a cellular assay that can examine the steps of cell-to-cell infection in T cells to identify therapeutics that are efficient in preventing this mode of transmission.

VS formation utilizes many cellular signaling and vesicular transport processes that could be targeted to inhibit infection. To screen for inhibitors, a desirable assay would measure the ability of HIV to engage in the early steps of viral replication up to the point of viral membrane fusion to identify inhibitors of HIV cell-to-cell transmission. A challenge to developing such an assay is that the VS requires direct mixing of infected and uninfected cells, limiting the sensitivity of conventional viral detection methods that measure the viral nucleic acids or gene products in both the donor and target cells within a mixed population. HIV reporter cell lines may be used, but these generally measure many steps associated with early infection including reverse transcription, integration and gene expression. An optimal system is one in which the expression of a heterologous marker gene is induced by the initiation of infection in the target cell but not present in the infected donor cell. The desired assay must be sensitive, without a high background signal. To address these design considerations, we have devised a system that utilizes a recombinant HIV that packages large amounts of the phage recombinase, Cre, into virus particles and uses a specially designed target cell that expresses a dsRed in the absence of recombination and GFP following Cre-mediated recombination. The resultant upregulation of GFP can be observed by fluorescence microscopy or quantified by flow cytometry.

To validate this assay, we have screened a small molecule library of 1998 drugs and compounds for inhibitors of HIV fusion. This library consists of compounds that affect a variety of cellular pathways and are categorized as 60% approved drugs, 25% natural compounds and 15% other bioactive compounds (less characterized toxins and cellular process inhibitors). Given the broad range of targets of this library, it may provide valuable information to identify unique structure–activity relationships required to inhibit HIV entry.

Results

Gag-iCre produces viral particles containing Cre recombinase

We set out to make HIV particles that package stoichiometric quantities of the Cre recombinase relative to the Gag polyprotein to serve as an indicator of viral entry when this enzymatic cargo is released into a target cell. The Cre gene was inserted into the gene for the HIV structural protein Gag, to create a virus analogous to a fluorescent virus that carried GFP in the same position (Hubner et al., 2007). The resulting virus packages an estimated 3000 molecules of Cre-recombinase within each virus particle (Briggs et al., 2004). The Cre is inserted between the matrix and capsid domains of Gag and flanked by viral protease sites so that the enzyme is proteolytically excised from the Gag precursor during HIV protease-mediated viral maturation (Fig. 1A). When viral membrane fusion occurs Cre within the virus particle is liberated into the target cell where it can activate recombination of a target substrate.

When transfected into 293T cells HIV Gag-iCre produces 60% less virus than a wild type NL4-3 HIV (Fig. 1B). Western blots on transfected 293T cells show that Cre is present both as part of a Gag precursor polyprotein and as a fully processed form in 293T producer cells (Fig. 1C Left). Western blot of the same lysates using an anti-HIV serum showed a larger p55 Gag-iCre polyprotein as well as fully processed p24 indicating that the insertion of Cre into the Gag polyprotein is compatible with processing of the Gag precursor to mature virus particles (Fig. 1C Right). In the virus particles produced by the HIV Gag-iCre virus, Cre protein was observed to be processed to lower molecular weight forms (Fig. 1D). Although HIV Gag-iCre does produce virus particles when transfected into 293T cells, we find that the HIV Gag-iCre virus is unable to mediate a spreading infection when infecting the highly permissive T cell line, MT4 (data not shown). Wild type virus showed an increase in p24 production peaking at 4 days after infection then decreasing, HIV Gag-iCre did not infect these cells when measured over the same time period (data not shown).

Gag-iCre reporter signal is blocked by inhibitors of fusion but not inhibitors of replication

A target cell line for the Cre virus called Jurkat floxRG was generated by transducing Jurkat cells with a reporter cassette containing dsRed fluorescent protein flanked by loxP recombination sites and followed by GFP (Koo et al., 2011). These cells were flow sorted for

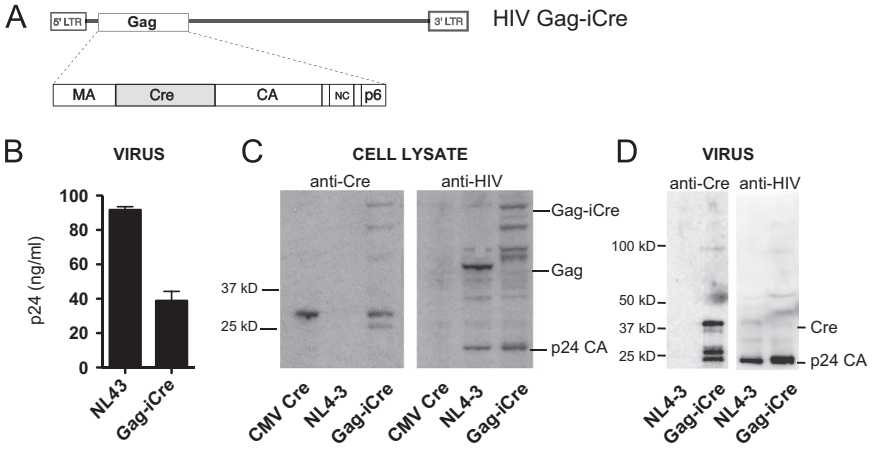

Fig. 1. HIV Gag-iCre carries the Cre gene inserted into HIV gag. (A) Insertion of Cre between the MA and CA domains allow high levels of Cre to be packaged into virus particles, with the Cre enzyme cleaved out of this precursor by the viral protease. (B) ELISA quantitation of p24 produced by Gag-iCre particles compared to NL4-3. (C) Western analysis of producer cell lysates using anti-cre antibody. (D) Western analysis of virus particles from supernatants using anti-cre antibody.