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Measuring HIV fusion mediated by envelopes from primary viral isolates

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ABSTRACT

Over the course of infection, the human immunodeficiency virus type 1 (HIV-1) continuously adapts in part to evade the host's neutralizing antibody response. Antibodies often target the HIV envelope proteins that mediate HIV fusion to its cellular targets. HIV virions pseudotyped with primary envelopes have often been used to explore the fusogenic properties of these envelopes. Unfortunately, these pseudotyped virions fuse with greatly reduced efficiency to primary cells. Here, we describe a relatively simple strategy to clone primary envelopes into a provirus and increase the sensitivity of the virion-based fusion assay.

1. Introduction

The human immunodeficiency virus (HIV) evolves rapidly, leading to an extensive genetic diversity within and between infected individuals. HIV diverged into several lineages or groups during multiple zoonotic infections occurring between nonhuman primates and humans (for review [1]). HIV type-1 (HIV-1) groups M, N, and O and HIV-2 appear to reflect different cross-species transmission events [2–5]. Group M has evolved into multiple subtypes due to adaptation and expansion in human hosts. These subtypes (or clades) are designated A through G and are joined in the pandemic by a few dozen additional strains that contain genetic segments derived from multiple subtypes, named circulating recombinant forms. HIV-1 subtypes share 70–90% sequence identity, groups share <70%, and HIV-1 and HIV-2 can differ by as much as 50% at the nucleotide level [6].

The genetic diversity of HIV-1 stems from the combination of point mutations and genetic recombinations [7]. Base substitutions are introduced principally by the error-prone reverse transcriptase [8] or by the mutagenic activities of host antiviral factors, such as the APOBEC3 (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) family of cytidine deaminases [9,10]. These processes introduce ~1 substitution per viral genome per generation. Genetic recombination results from the migration of reverse transcriptase between the two viral RNA templates encapsidated in the virion and happens several times in any given round of HIV-1 DNA synthesis. Both the incorporation of two heterologous genomic RNAs and recombination-prone replication machinery are critical for this high frequency of recombination. The vast sequence variability of the HIV-1 virus allows escape variants to arise upon selective pressure exerted by the immune system or antiviral drugs.

One of the most variable genes of HIV is the envelope (*env*). The *env* gene encodes a precursor, gp160, that is cleaved by the cellular protease furin into two subunits, gp120 and gp41. These proteins remain associated at the cell surface due to electrostatic interactions. Gp120 and gp41 heterodimers are anchored on the viral particle by the transmembrane domains of the gp41 subunit and assemble into trimeric structures to form the viral spike. Viral cell entry is initiated by the binding of gp120 to the CD4 receptor. Later binding of gp120 to the CCR5 or CXCR4 coreceptor [11] induces conformational changes that trigger the activation of gp41 [12–14] and leads to fusion of virions to the target cell [12,15].

Gp120 consists of five highly variable regions (designated V1–V5) that are interspersed between five more conserved regions (C1–C5) [16]. The variable loops shield the more conserved regions that mediate binding to the receptors [17]. When gp120 binds to CD4, structural changes expose previously masked epitopes and surfaces [18,19]. V1, V2, V4 and V5 are characterized by rapid changes in the length, number and localization of glycosylation sites [20,21].

Abbreviations: MDDCs, monocyte-derived dendritic cells; PBMCs, peripheral blood mononuclear cells; PBLs, peripheral blood lymphocytes; BlaM, beta lactamase.

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Because of the extreme genetic diversity of HIV-1 *env*, it is important that functional studies of HIV-1 envelopes be performed with primary rather than laboratory-adapted gene products. We previously described an HIV virion-based fusion assay that is able to measure the properties and kinetics of fusion of laboratory-adapted viruses to a wide variety of target cells, including biologically relevant primary cells. Unfortunately, this assay performs less efficiently when virions are pseudotyped with primary envelopes [22]. Thus, this shortcoming limited the type of target cells that could be studied as well as the analysis of fusion induced by various primary envelope proteins. Here, we describe the construction of viruses encoding primary envelopes in *cis* and their successful use to study the fusogenic properties of various primary HIV envelope proteins.

2. Materials and methods

2.1. Proviral DNA and HIV envelopes

pCMV4-BlaM-Vpr is available upon request at Addgene (Cambridge, MA). pAdVAntage is a commercially available construct (Promega, Madison, WI). The proviral constructs pNL4-3ΔEnv and TN6-GFP are as described [23] and [24]. The pCR3.1 vectors encoding the primary envelopes 55FPB28a and 109FPB4 are as described [25]. The vectors expressing primary HIV envelope proteins (pSVIII-92RW020.5, pSVIII-92HT599.24, pSVIII-93MW965.26, pSVIII-92UG021.16) were obtained from the NIH AIDS Research & Reference Reagent Program [26].

2.2. Cloning the primary envelope into the TN6-GFP vector

To facilitate cloning of the primary envelopes into the proviral DNA, we selected the TN6-GFP proviral DNA expression vector, an NL4-3-based construct modified to contain a BstEII restriction site 15 nucleotides (nt) after the signal peptide of NL4-3 *env* and a NcoI site at the end of the envelope (for map see Fig. 1 [24]). Primary envelopes were amplified with the sense primer C6323+ as described [24] (ttgtgGGTCACCgtctattatgggg) and the anti-sense primer ASenvNcoI (ctgcatCCATGGtttattgtaaagctgcttc). The PCR amplification was performed in 50 μl of a solution containing 100–250 ng of purified vector encoding the envelopes, 20 pmol of each primer, 200 μM dNTPs, and 1× buffer containing 15 mM $MgCl_2$, and 2.6 U of Taq DNA polymerase (Expand High Fidelity PCR System, Roche). The PCR parameters were 94 °C for 2 min to achieve initial denaturation, followed by 30 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 3 min and a final elongation at 72 °C for 30 min. The PCR products were analyzed on 1% agarose gels, purified using QIAquick kit (QIAGEN, Valencia, CA) and subcloned into the TOPO XL vector (Invitrogen, Carlsbad, CA). To release the insert, the TOPO clones were then digested by BstEII and NcoI (NEB, Ipswich, MA). After gel purification, these inserts were ligated using T4 ligase (NEB, Ipswich, MA) into TN6-GFP previously cut with BstEII and NcoI. Ligation was performed in 20 μl of a solution containing 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 25 μg/ml bovine serum albumin, and 2000 U of T4 DNA ligase (NEB). Use of approximately 3 inserts per 1 proviral vector yielded high levels of ligation. To further increase the ligation efficiency, temperatures were alternated between 16 and 37 °C every 30 s. Half of the ligation products (i.e., 10 μl of the ligation reaction) were used to transform MAX Efficiency Stbl2 competent cells (Invitrogen). The resulting TN6-GFP clones containing the primary envelopes were then amplified and purified using a QIAGEN plasmid mega kit. Sequences were confirmed by sequencing.

2.3. Generation and culture of PBLs and MDDCs

Peripheral blood mononuclear cells (PBMCs) were purified from fresh buffy coats on Ficoll gradients. $CD14^+$ monocytes were positively selected using Miltenyi anti-CD14 magnetic beads, according to the manufacturer's instructions. DCs were derived by culturing $CD14^+$ monocytes (2×10^6 cells/ml) for 6 days with 25 ng/ml IL4 (R&D Systems, Minneapolis, MN) and 50 ng/ml GM-CSF (Biosource, Camarillo, CA) in RPMI medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin [27]. The medium was changed every 2 days. Autologous peripheral blood lymphocytes (PBLs) were maintained in RPMI medium until used in the fusion assay in parallel to the MDDCs.

2.4. Production of NL4-3 virions containing BlaM-Vpr

HIV virions containing BlaM-Vpr were produced by transfection of 293T cells with calcium phosphate-induced DNA precipitates [28,29]. 293T cells (1.5×10^7) were plated in 40 ml of DMEM culture medium in a T175 cm^2 tissue-culture flask and cultured overnight at 37 °C in a 5% CO_2 humidified incubator. The medium was then exchanged with 40 ml of DMEM pre-warmed to 37 °C. The DNA (60 μg of TN6-GFP proviral DNA, 20 μg of pCMV-BlaM-Vpr and 10 μg of pAdVAntage vectors) was diluted in 1.75 ml of H_2O, and precipitates were formed by successively adding 2 ml of 2× HBS (280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM dextrose, 50 mM Hepes, pH 7.05), and 250 μl of 2 M $CaCl_2$ drop by drop. After 30 min of incubation at room temperature, the precipitate was added dropwise to the 293T cells. Pseudotyped viruses were produced by transfection of 40 μg of NL4-3ΔEnv proviral DNA, 20 μg of envelope-expressing constructs, 20 μg of pCMV-BlaM-Vpr and 10 μg of pAdVAntage vector DNA. After 16 h at 37 °C, the medium was replaced with 20 ml of fresh DMEM complete medium pre-warmed to 37 °C. Viral production was allowed to continue for 24 h. Virion-containing supernatants were harvested and centrifuged at 800*g* at room temperature for 10 min to remove cellular debris. Virions were next pelleted by ultracentrifugation at 72,000*g* at 4 °C for 90 min. The viral pellet was then resuspended in 400 μl of DMEM, aliquoted and stored at −80 °C. The $p24^{Gag}$ content of the viral preparations was quantified with the Alliance HIV-1 p24 ELISA Kit (Perkin–Elmer), according to the manufacturer's instructions. Virion yield after ultracentrifugation was 50–100 μg of $p24^{Gag}$/ml.

2.5. HIV-1 virion fusion assay

The virion-based fusion assay was described in considerable detail [28,29]. This assay involves three steps: (1) incubation of target cells with NL4-3 virions containing BlaM-Vpr, (2) loading of target cells with the CCF2-AM substrate and development of the BlaM reaction, and (3) immunostaining and fixation. 5×10^5 MDDCs or 2×10^6 PBLs were infected with HIV virions containing BlaM-Vpr (equivalent of 500 ng of $p24^{Gag}$) for 2 h at 37 °C in a volume of 100 μl. For convenience, the infection was performed in a V-bottom 96-well plate. After infection, the cells were washed once in CO_2-independent DMEM medium and loaded with the CCF2-AM substrate (Invitrogen) by incubating the cells for 1 h at room temperature in a solution of CO_2-independent medium containing 1 μM CCF2-AM and a dilution 1/100 of the solution B provided by Invitrogen (100 mg/ml Pluronic-F127 and 0.1% acetic acid). The cells were next washed again in CO_2-independent media containing 10% FBS and incubated overnight in 200 μl of CO_2-independent media containing 10% FBS and 250 mM probenecid, an inhibitor of anion transport, at room temperature and in the dark. For the analysis of fusion to CD4 T-cell PBLs, the fusion assay was combined with an immunostaining with anti-human CD3 conju-