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A virus–envelope paired competitive assay to study entry efficiency of human immunodeficiency virus type 1 *in vitro*

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The efficiency of the human immunodeficiency virus type-1 (HIV-1) to enter cells is defined primarily by amino acid exchanges in the external glycoprotein gp120 and in, especially its highly variable V3 loop region. To study entry efficiency of HIV-1 a competitive viral entry assay was developed, to be comprised of infectious virus as well as soluble gp120 (sgp120) as an entry competitor.

Entry of viruses using the coreceptor CXCR4 was reduced by adding CXCR4-tropic sgp120 (X4-sgp120) SF2 or LAV expressed in the baculovirus system or by adding X4-sgp120 from NL-952 and NL-V3A virus mutants produced in a HeLa-P4 cell culture expression system. Adding X4-sgp120 into a CCR5-specific infection assay revealed that X4-sgp120 enhanced the infection of CCR5-tropic virus. Furthermore, the role of the V3 loop N-glycan g15 on entry efficiency was studied using virus mutants and sgp120 with different N-glycosylation and different coreceptor usage. These experiments showed that viral entry of R5-tropic viruses lacking the N-glycan g15 within the V3 loop was inhibited by CCR5-tropic sgp120 harboring the g15 N-glycan.

Altogether, the data demonstrate that HIV-1 entry efficiency can be studied easily by using sgp120 as an internal control or by using autologous or heterologous sgp120–virus pairs.

1. Introduction

The role of the human immunodeficiency virus type-1 (HIV-1) external glycoprotein gp120 is the binding to specific receptors on target cells to initiate the process of viral entry. The receptor molecules needed are CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984) and one of the two coreceptors CCR5 and CXCR4 (Alkhatib et al., 1996; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996). The efficiency of virus entry is determined mainly by genetic variation in the *env* gene, especially in the highly variable V3 loop a domain of the external glycoprotein gp120 (Reeves et al., 2004; Biscone et al., 2006; Lobritz et al., 2007). Amino acid exchanges which affect V3 loop length, loop conformation, N-glycosylation and the overall positive charge, altogether have a significant influence on CCR5 or CXCR4 coreceptor usage. Therefore, HIV-1 V3-variants differ in coreceptor interaction and as a consequence in their efficiency to enter target cells (Polzer et al., 2001, 2002). In addition to the V3 loop, regions in gp120 including V1 (Hamoudi et al., 2013), V2 (Saunders et al., 2005; Wolk and Schreiber, 2006) and C4 (Monno et al., 2011), were also investigated as potential determinants involved in the viral entry process.

Viral entry efficiency can be monitored by either a monoinfection assay (Quiñones-Mateu and Arts, 2002, 2006) or a pairwise growth competition assay (Quiñones-Mateu et al., 2000; Ball et al., 2003). Culture and growth conditions for monoinfection virus-cell based assays are in principle carried out without an internal control. Since virus infection assays are also slightly inconsistent, results of monoinfection assays might not reveal relevant minor changes in viral entry efficiency. On the other hand, the improvement of an infection assay by adding an internal virus reference in form of a known HIV-1 strain, adds an extra viral growth kinetic to the assay that could significantly influence the growth of the unknown virus. Such a competitive assay should ideally imitate the situation where multiple variants of HIV-1 are in rivalry for binding to cellular receptors. But in a closed, cell-limited in vitro culture system the starter conditions for each virus have an important impact on the particular outcome (Lanxon-Cookson et al., 2013).

Although several monoinfection and competitive assays have been developed to study viral fitness, a test that is focused mainly on *env*-based entry effects is not available. In the present study a

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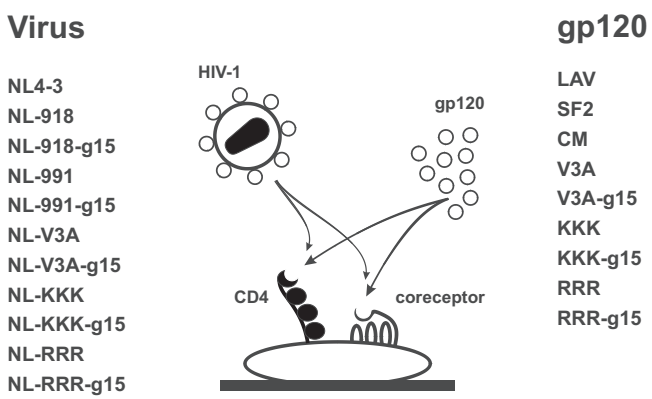

Fig. 1. Design of the experiments. The experiment is a virus infection assay using TZM-bl cells which express the viral receptors CD4, CXCR4, CCR5 and additionally a reporter gene for β-gal that is controlled by the HIV-1 LTR. As a competitor for viral entry soluble forms of gp120 are added to the virus supernatant and the virus–gp120 mixture was used for TZM-bl infection. Infection rates were monitored by β-gal staining of HIV-1 positive cells 48 h post infection. The viruses (NL-constructs) and envelopes (sgp120) used for the experiments are indicated and their corresponding V3 loop sequences are shown in Table 1. The NL4-3 V3 loop virus mutants were all constructed as described by Polzer et al. (2001, 2002) using the virus vector pNL4-3-Bst. For sgp120, purified envelope proteins LAV (baculovirus origin), SF2 (CHO cell origin) and CM (baculovirus origin) were used. All other gp120 proteins were produced in the human HeLa-P4 cell line and applied into the infection assay in form of diluted cell culture supernatants.

simple test for the comparison of viral entry efficiency and the study of *env* based effects on viral infection by using sgp120 is described. A general overview of the experimental design as well as the viruses and envelope glycoproteins used in this study is shown in Fig. 1.

The present study demonstrates that the entry of X4-tropic viruses was reduced when X4-tropic sgp120 from HIV-1 strains SF2 or LAV was added. Thus, free X4-tropic sgp120 was competitive for receptor binding and able to prevent X4-tropic virus entry. In contrast, the entry efficiency of R5-tropic viruses was enhanced by X4-tropic sgp120. A role of the V3 loop N-glycan g15 for entry efficiency was also demonstrated. Infection of R5-tropic viruses lacking N-glycan g15 (−g15) was reduced by the corresponding +g15 sgp120 proteins. In addition, X4-tropic mutants were constructed only differing in the positively charged amino acids lysine and arginine. The KKK-mutants were enhanced significantly by RRR-sgp120 in a heparan sulfate proteoglycan dependent manner. Taken together, entry of HIV-1 can be modified significantly by sgp120 from a different virus mutant, with differences in coreceptor usage, entry efficiency or N-glycosylation. Besides the practical approach of the gp120–virus competition assay, the assay might also give new insights in the complex virus–cell interplay and the evolutionary forces driving the intra-patient evolution of the HIV-1 quasispecies.

2. Materials and methods

2.1. Soluble gp120

Recombinant, full length, glycosylated and biological active sgp120 from HIV-1 strains LAV and CM (corresponding to HIV-1 strain CM243) were obtained from Protein Sciences Corporation (Meriden, USA) and SF2 sgp120 was obtained from the Centre for AIDS Reagents at the National Institute for Biological Standards and Control (No. ARP629, Hertfordshire, UK). To generate sgp120 corresponding to the NL4-3 virus V3 loop mutants, the *env* genes were cloned into the gp160 expression vector pSVAT-Grev as described earlier (Polzer et al., 2010). Soluble gp120 was produced by FuGENE®6 (Promega, Mannheim, Germany) transfection of 3×10^4 HeLa-P4 cells, a HeLa derivative expressing CD4 (Clavel and Charneau, 1994), with 1 μg pSVATGrev plasmid DNA, representing the gp120 V3 loop mutants (see Table 1). The cell culture supernatants were collected after three days and cleared from cell debris by centrifugation (Eppendorf centrifuge model 5430R, 12.000 rpm).

2.2. gp120 dot blot assay

Concentration of sgp120 in the HeLa-P4 cell culture supernatants was monitored by an in-house dot blot assay using the D7324 gp120-specific alkaline phosphatase-conjugated monoclonal antibody (Aalto Bio Reagents, Dublin, Ireland) and LAV sgp120 as a control (Protein Sciences Corporation, Meriden, USA) (Fig. 2). One microliter of the cell culture supernatants and 1 μl of the LAV sgp120 controls (250, 125, 63, 32 and 16 ng/ml) were dotted directly onto a nitrocellulose test strip (BA-85, Schleicher & Schuell, Dassel, Germany). After immobilization the membrane was blocked with NTTM buffer (100 mM NaCl; 10 mM Tris HCl pH 7.5; 0.05%, Tween20; 10% low fat milk) for 30 min at RT. Anti-gp120 antibody was diluted 1:1000 in NTTM buffer and incubated with the test strip for 1 h at room temperature. The test strip was washed three times with NTT buffer (100 mM NaCl; 10 mM Tris HCl pH 7.5; 0.05%, Tween20) and than again three times with NT buffer (100 mM NaCl; 10 mM Tris HCl pH 7.5). Bound antibody was detected by staining with a one-step AP NBT (nitro-blue tetrazolium)/BCIP (5-bromo-4-chloro-3′-indolyphosphate) substrate solution (Thermo Fisher Scientific, Bonn, Germany). After 15 min the test strips were rinsed with water and dried prior to image scanning. The software ImageJ was a public domain software distributed by Wayne Rasband

Table 1
V3 sequences of gp120 and NL4-3 mutants.

	V3 loop amino acid sequence	Coreceptor usage	V3 charge	Reference
NL4-3/LAV	CTRPNNNTRKSIRIQRGPGRAFVTIGKIGNMRQAHC	X4	8	Gene accession U26942
SF2	CTRPNNNTRKSIYIGPGRAFHTTGRIIGDIRKAHC	X4	6	Gene accession K02007
CM	CTRPSNNTRPSITVGPGQVFYRTGDIIGDIRRAYC	R5	3	Gene accession L03703
NL-918	CTRPSNNTRKSIHIGPGRAFYATGEIIGDIRQAHC	R5	3	Polzer et al. (2001)
NL-918-g15	CTRPS**Q**NTRKSIHIGPGRAFYATGEIIGDIRQAHC	R5	3	Polzer et al. (2001)
NL-991	CTRPNNNTRRSIPIGPGRAFYTTGDIVGDIRQAHC	R5	3	Polzer et al. (2001)
NL-991-g15	CTRPN**Q**NTRRSIPIGPGRAFYTTGDIVGDIRQAHC	R5	3	(Polzer et al. (2001)
NL-V3A	CIRPNNNTRKSVRIGPGQAFYATGDIIGNIRQAHC	R5	4	n.a.
NL-V3A-g15	CIRPN**Q**NTRKSVRIGPGQAFYATGDIIGNIRQAHC	R5	4	n.a.
NL-KKK	CTRPNNNT**KKK**ITLGPGRVLYTTGEIIGDIRKAHC	R5X4	5	Polzer et al. (2002)
NL-KKK-g15	CTRPN**Q**NT**KKK**ITLGPGRVLYTTGEIIGDIRKAHC	X4	5	Polzer et al. (2002)
NL-RRR	CTRPNNNT**RRR**ITLGPGRVLYTTGEIIGDIRKAHC	X4	5	Polzer et al. (2002)
NL-RRR-g15	CTRPN**Q**NT**RRR**ITLGPGRVLYTTGEIIGDIRKAHC	X4	5	Polzer et al. (2002)