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A high-throughput assay for HIV-1 integrase 3′-processing activity using time-resolved fluorescence

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A B S T R A C T

HIV-1 integrase (HIV-1 IN), a well-validated antiviral drug target, catalyzes multistep reactions to incorporate viral DNA into the genome of the host cell; these include both a 3′-processing (3′P) reaction and a strand transfer reaction. These enzymatic activities can be measured in vitro with short DNA oligonucleotides that mimic a single viral LTR DNA end and purified IN. A highly sensitive and reproducible time-resolved fluorescence (TRF)-based assay for HIV-1 IN 3′P activity is now reported. This assay was optimized with respect to time and concentrations of metal ions, substrate and enzyme. The assay has now been used successfully to measure HIV-1 IN 3′P activity and has been shown to detect the anti-IN activity of several known 3′P inhibition compounds accurately. This assay, which is amenable to high-throughput screening, will be useful for identification of additional HIV-1 IN 3′P inhibitors.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) integrase (IN) is an essential enzyme in the viral cycle that functions by inserting the viral genome into the genome of infected cells (Pommier et al., 2005; Li et al., 2011). Thus, IN is an attractive, validated target for the development of new HIV antiviral drugs, because IN has no cellular homologue in human (Marchand et al., 2009). HIV-1 IN catalyzes two major reactions: an endonucleolytic cleavage known as 3′-processing (3′P), in which a dinucleotide is excised from each end of the viral LTR DNA, and a strand transfer reaction leading to the insertion of the processed viral DNA into the target host DNA. Each of these activities has been demonstrated in vitro by making use of short duplex oligonucleotides (18–35 bp) that mimic the U5 or U3 LTR ends and recombinant IN (John et al., 2005).

In the search for antiviral compounds that target HIV-1 IN, convenient and high-throughput methods for measuring enzymatic activity are desirable. Moreover, the development of such assays is also critical to define quantifiable structure-activity relationships of antiviral compounds. Several in vitro biochemical assays have been reported that measure the enzymatic activity of HIV-1 IN alone and in the presence of IN inhibitors. Some of these assays measure 3′P and strand transfer activities together, whereas others measure 3′P activity alone. Traditionally, integrase activities have been measured by low-throughput gel-based assays involving radioactively labeled oligonucleotides (Debyser et al., 2001). Although this assay is still commonly used, it is laborious and time-consuming; it is also inconvenient because it involves the handling of hazardous radioactive waste. Therefore, there is great interest in the development of non-radioisotopic high-throughput assays for measuring HIV-1 IN activity and a number of microtiter plate assays have been described (Craigie et al., 1991; Hazuda et al., 1994; Vink et al., 1994; Hawkins et al., 1995; John et al., 2005).

More recently, several fluorescence-based assays have been reported to measure the 3′P activity of the HIV-1 IN. A fluorescence resonance energy transfer (FRET)-based assay that can measure both the 3′P and strand transfer activities of HIV-1 IN has been used for high-throughput screening of HIV-IN inhibitors (Wang et al., 2005). This assay used an oligonucleotide substrate dually labeled with biotin and the fluorescent dye Cy5, and it combines time-resolved fluorescence (TRF) with FRET. This assay has the advantage of avoiding plate coating and wash steps but requires

Abbreviations: BSA, bovine serum albumin; DELFIA, dissociation-enhanced lanthanide fluoroimmunoassay; DMSO, dimethyl sulfoxide; DTPA, diethylenetriaminepentaacetic acid; FRET, fluorescence resonance energy transfer; HIV-1 IN, HIV-1 integrase; IPTG, isopropyl β-D-1-thiogalactopyranoside; 3′ P, 3′ processing; LTR, long terminal repeat; PBS, phosphate buffered saline; TRF, time-resolved fluorescence.

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multiple labeling of the oligonucleotide substrate and an advanced spectrometer with a dichroic mirror for dual fluorescence detection. Two different high-throughput assays have also been reported to measure HIV-1 IN 3′P activity (He et al., 2007; Fenwick et al., 2011). However, these methods are limited by the need for large amounts of oligonucleotide substrate and/or a low signal-to-noise ratio. In addition, a fluorescence anisotropy assay has been recently reported (Guiot et al., 2006; Merkel et al., 2009) but requires advanced technologies that are not available in most laboratories.

TRF, in which a short delay between excitation and detection of emitted light diminishes any autofluorescence of substrate or background, has a low signal-to-noise ratio. On the basis of TRF detection, a dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA), developed by PerkinElmer Life Science, further improves sensitivity and represents a sensitive and reliable platform (Dickson et al., 1995). A robust TRF-based assay is herein described for detection of HIV-1 IN 3′P activity that permits the study of enzyme kinetics and high-throughput screening of anti-HIV-1 IN agents.

2. Materials and methods

2.1. Materials

Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, USA): (I) 5′-amino-C12-ACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGT-3′ (34-mer), (II) 5′-ACTGCTAGAGATTTTCCACACTGACTAAAAG-3′ (31-mer). For oligonucleotide annealing, oligo I + II (3P substrate) were mixed at a molar ratio of 1:1 at 50 μM in 10 mM Tris (pH 7.8), 0.1 mM EDTA, heated to 95 °C for 5 min, slowly cooled down to room temperature, and stored at −20 °C till use. For the standard radioactive gel assay, a pair of 21-mer oligonucleotides, i.e. INT1: 5′-GTGTGGAAAATCTCTAGCAGT-3′ and INT2: 5′-ACTGCTAGAGATTTTCCACAC-3′ were used. Primers used for mutagenesis in regard to an enzymatically inactive form of IN containing a D116A substitution were: 5′-CAGTAAAAACAGTACATACAGCCAATGGCAGCAATTTCACCAG-3′ (D116A-sense) and 5′-CTGGTGAAATTGCTGCCATTGGCTGTATGTACTGTTTTTACTG-3′ (D116A-antisense).

DNA-BIND™ 96-well plates containing N-oxysuccinimide ester groups to permit binding of viral LTR were purchased from Corning (Lowell, MA, USA). DELFIA Eu-labeled streptavidin (SA-Eu chelate) enhancement solution and γ-[^{32}P]-ATP were purchased from PerkinElmer (Waltham, MA, USA). EXO900 was obtained from Boehringer Ingelheim (Canada) (Laval, QC, Canada). Raltegravir (RAL) was obtained from Merck-Frosst Canada (Kirkland, QC, Canada). Dolutegravir (S/GSK1349572) was obtained from Shionogi/ViiV Healthcare (North Carolina, USA). Baicalein, myricetin, bovine serum albumin (BSA) and diethylenetriaminepentaacetic acid (DTPA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were purchased from BioShop Canada (Burlington, Ontario, Canada).

2.2. Integrase purification

The D116A mutation was introduced by site-directed mutagenesis into the HIV-1 integrase gene using the *Pfu* Turbo Hotstart DNA polymerase (Stratagene) according to the manufacturer's recommendations. The presence of the mutation was confirmed subsequently by sequencing. Recombinant wild-type (WT) IN and the mutated D116A-containing IN enzyme were expressed in *Escherichia coli* BL21 (DE3) and purified from the soluble fraction as described previously (Bar-Magen et al., 2009) with minor modifications. Briefly, the integrase gene was cloned into pET15B plasmid allowing the expression of N-terminus 6-His tagged protein. Cells were grown to mid-logarithmic phase in Luria-Bertaini medium and induced for 3 h with 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside). Cells were centrifuged, resuspended in lysis buffer (20 mM Hepes pH7.5, 100 mM NaCl, 2 mM DTT) and sonicated for 1 min on ice, followed by centrifugation for 30 min at 12,000 g. The pellet was re-extracted once more in binding buffer (20 mM Hepes pH7.5, 20 mM imidazole, 1 M NaCl, 2 mM DTT) and sonicated for 1 min on ice, followed by centrifugation. The two supernatants were collected, pooled, and purified using Ni-NTA agarose (Qiagen). After washing, proteins were eluted using elution buffer (25 mM Hepes pH7.5, 10% glycerol, 1 M NaCl, 600 mM imidazole). Fractions containing integrase were identified using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing purified integrase were pooled and dialyzed into storage buffer (20 mM Hepes, pH 7.5, 1 M NaCl, 1 mM EDTA, 10% glycerol, 5 mM DTT) using a 12-mL Slide-A-Lyzer, 20,000 MWCO (Thermo Scientific, Rockford, IL). Aliquoted protein fractions were stored at −80 °C. Protein concentrations were measured at A280 and calculated using an extinction coefficient of 50,420, and confirmed by the Bradford protein assay. Then, equal amounts of proteins were verified by SDS-PAGE. The purified INs were free of nuclease contamination as verified by a nuclease assay.

2.3. Pretreatment of microplates

The annealed LTR duplex was diluted to suitable concentrations in PBS (pH 7.4) and pipetted in 80 μL aliquots into the wells of DNA Bind™ 96-well plates (Corning). As negative controls, the wells in the first row of the plate were coated with PBS alone without any LTR. The coated plates were kept at 4 °C overnight. After discarding the coating solution, the plates were then blocked with 100 μL 1% BSA in blocking solution (20 mM Tris, pH 7.5, 150 mM NaCl) at 4 °C overnight, and kept at 4 °C in blocking solution until use.

2.4. Microplate assay of HIV-1 3′P activity

First, the coated plates were washed with PBS and assay buffer (50 mM MOPS pH 6.8, 50 mM NaCl, 15 mM $MnCl_2$, 50 μg/mL BSA, 0.15% CHAPS) twice, respectively. 3′P assays were performed directly in these oligonucleotide-coated microtiter wells using the immobilized LTR. Then, 100 μL of purified HIV-1 IN were added into each well, except for those wells that were set as a reference for the total immobilized LTR. Subsequently, the reaction was initiated by incubation at 37 °C for 2 h or as otherwise stated. After the reactions were completed, the plates were washed 3 times with 250 μL wash buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20, 2 mg/mL BSA) to remove the cleaved biotinylated dinucleotide. After 150 μL Eu^{3+}-SA (0.025 μg/mL) in wash buffer containing 50 μM DTPA was added to each well, the plates were incubated for 30 min at room temperature. Then, the plates were washed again with wash buffer four times, and 100 μL of DELFIA enhancement solution was added to the wells. The plates were allowed to stand for 15 min at room temperature. TRF signals were measured at an excitation wavelength of 340 nm and an emission wavelength of 615 nm, with 400 μs lag, using a FLUOStar Optima plate reader (BMG Labtech).

Substrate-free control wells were analyzed under the same reaction conditions without immobilizing LTR in the wells to monitor the background signal ($3P_{background}$). Enzyme-free control wells were set with all reagents except for enzyme to measure the total immobilized LTR substrate as a reference ($3P_{control}$). Reactions with immobilized LTR substrate under different conditions were performed to measure the remaining uncleaved LTR substrate ($3P_{substrate}$). The 3P activity for each sample was calculated using the following equation:

$$3P_{sample} = [(3P_{control} - 3P_{background}) - (3P_{substrate} - 3P_{background})].$$