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Research paper

Biochemical properties of the xenotropic murine leukemia virus-related virus integrase

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ABSTRACT

Xenotropic Murine Leukemia Virus-related Virus (XMRV) is a new gammaretrovirus generated by genetic recombination between two murine endogenous retroviruses, PreXMRV1 and PreXMRV2, during passaging of human prostate cancer xenografts in laboratory mice. XMRV is representative of an early founder virus that jumps species from mouse to human cell lines. Relatively little information is available concerning the XMRV integrase (IN), an enzyme that catalyzes a key stage in the retroviral cycle, and whose sequence is conserved among replication competent retroviruses emerging from recombination between the murine endogenous PreXMRV-1 and PreXMRV-2 genomes. Previous studies have shown that IN inhibitors efficiently block XMRV multiplication in cells. We thus aimed at characterizing the biochemical properties and sensitivity of the XMRV IN to the raltegravir, dolutegravir, 118-D-24 and elvitegravir inhibitors *in vitro*. We report for the first time the purification and enzymatic characterization of recombinant XMRV IN. This IN, produced in *Escherichia coli* and purified under native conditions, is optimally active over a pH range of 7–8.5, in the presence of Mg^{2+} (15 mM and 30 mM for 3′-processing and strand transfer, respectively) and is poorly sensitive to the addition of dithiothreitol. Raltegravir was shown to be a very potent inhibitor (IC_{50} ~ 30 nM) whereas dolutegravir and elvitegravir were less effective (IC_{50} ~ 230 nM and 650 nM, respectively). The 118-D-24 drug had no impact on XMRV IN activity. Interestingly, the substrate specificity of XMRV IN seems to be less marked compared to HIV-1 IN since XMRV IN is able to process various donor substrates that share little homology. Finally, our analysis revealed some original properties of the XMRV IN such as its relatively low sequence specificity.

1. Introduction

Xenotropic Murine Leukemia virus-related Virus (XMRV) is a new gammaretrovirus with tropism for human cells that originated from a genetic recombination between two murine endogenous retroviruses, PreXMRV-1 and PreXMRV-2, carried in the germline DNA of laboratory mice used during serial transplantations of CWR22 prostate cancer xenografts [1]. Originally identified in human prostate tumor tissues using a Virochip DNA array [2], this virus does not circulate in humans, but has jumped across species to contaminate human cell lines and patients' biopsies in independent laboratories (reviewed in Ref. [3]). This widespread contamination however questions biological safety and the risk associated with a replication-competent potentially hazardous new virus capable of infecting human cells. In particular, XMRV presents a highly oncogenic potential due to its preferred genotoxic integration site [4–6]. Fortunately, the XMRV cycle seems to be efficiently restricted [7] notably in primary human cells [8].

Emergence of recombinant replication competent retroviruses (RCRs) related to xenotropic murine leukemia virus variants (X-MLV isolates) during xenografts is a frequent phenomenon [9,10]. Studies on XMRV and other X-MLVs could thus provide clues on how a new retrovirus could circumvent barrier species. Therefore,

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emergence of RCRs from recombination between PreXMRV-1 and PreXMRV-2 has been recently reproduced *in vitro* [11]. The RCRs obtained during this study are not strictly identical to XMRV but, as do XMRV, they all possess U3, U5 and integrase (IN) sequences identical or closely related to those of PreXMRV-1 [11], suggesting that these sequences have been positively selected over PreXMRV-2 sequences during serial infections both *in vitro* or *in vivo*. Concerning the U3 region, this sequence is poorly adapted to human cells, driving the transcription at a level similar to that of a Tat-defective HIV-1 [12]. Despite this low fitness, XMRV can efficiently be propagated through the huge accumulation of viral copies (≈40 copies per cell) that compensates for the low expression level of individual proviruses [12]. Therefore, one could hypothesize that the strong conservation of the PreXMRV-1 IN and its long terminal repeat (LTR) substrates might rather be linked to IN activity.

Several studies already investigated XMRV susceptibility to antiretroviral inhibitors [13–16]. XMRV appeared to be intrinsically resistant to several drugs used to treat HIV-1 infection including some non-nucleoside reverse transcriptase (RT) inhibitors (nevirapine, efavirenz, a TIBO derivative) [15,16], the pyrophosphate analog foscarnet and all FDA-approved inhibitors of HIV-1 protease [15,16]. This may be due to structural differences in their respective enzymes. However, XMRV multiplication is efficiently blocked by a subset of nucleoside analog inhibitors (including AZT, tenofovir) [13,14,16], and by the IN strand-transfer inhibitors (INSTI), raltegravir, L-000870812 and elvitegravir [15,16]. Notably, of the 24 and 45 antiretroviral drugs tested by Smith et al. [16] and Singh et al. [15], respectively, raltegravir was the most potent anti-XMRV drug, with EC_{50} (median effective concentration) values at nanomolar concentrations [14–16]. Interestingly, despite differences in their respective amino acid sequences, HIV-1 and XMRV IN showed comparable EC_{50} values for raltegravir, suggesting that they share similar inhibitor-binding surfaces. Elvitegravir was a less potent XMRV IN inhibitor with an EC_{50} at least 40-fold greater relative to raltegravir. These observations are in agreement with a previous study reporting that retroviruses from the alpha-, beta-, gamma- and lentiviral genus were sensitive to raltegravir whereas elvitegravir was more active against lentiviruses [17]. Of note, Sakuma et al. [13] reported that the IN inhibitor, 118-D-24, was ineffective against XMRV.

In vivo, IN is part of the preintegration complex formed between viral DNA and proteins of viral or cellular origins. This enzyme catalyzes the essential reaction of integration of a proviral cDNA copy into the host genome, a step required to ensure efficient expression and replication of the viral genome. At least two activities are central for the integration process. First, the IN removes two 3′-nucleotides from each strand of the linear viral DNA, a process named 3′-processing that results in overhanging CA ends. Second, during the strand transfer reaction, IN catalyzes the nucleophilic attack by the 3′-processed ends of phosphodiester bonds on the opposite strand of the target DNA (for a review see Ref. [18]). In addition to 3′-processing and strand transfer, IN may catalyze other reactions *in vitro* including disintegration (apparent inverse reaction of strand transfer) and palindrome cleavage [18,19].

To characterize one of the major targets of anti-XMRV drugs, we report the analysis of catalytic properties and sensitivity to inhibitors of the recombinant XMRV IN. This study defined optimal *in vitro* conditions for XMRV IN activity, and provides a comparative analysis of the XMRV and HIV-1 INs. We also characterized conditions in which 3′-processing and strand transfer activities can be selectively dissociated. Finally, our analysis revealed some original properties of PreXMRV-1/XMRV IN such as its low primary sequence specificity *in vitro*.

2. Materials and methods

2.1. Double stranded oligonucleotide substrate preparation

Oligonucleotides (Table S1) were purchased from Eurogentec and purified as previously described [20] by electrophoresis in a denaturing 16% acrylamide (19:1)/5.6 M urea gel. The nomenclature of oligonucleotide duplex substrates (Table S1) is the following: “X” and “H” designate XMRV and HIV-1 sequences, respectively; “b” indicates that the substrate mimics a blunt extremity; “p” indicates that the substrate mimics a processed extremity. Oligonucleotides corresponding to the end of the U5 or U3 LTRs of the viral genome were radiolabeled with T4 polynucleotide kinase (NEB, France) and [γ-^{32}P] ATP (3000 Ci/mmol) (Perkin Elmer, France), then purified on a Sephadex G-25 column (GE Healthcare, USA). Double-stranded oligonucleotides were obtained by mixing equivalent molar amounts of complementary strands in the presence of 100 mM NaCl, heating at 95 °C for 5 min and slowly cooling to room temperature.

2.2. XMRV IN cloning

The pCDNA3.1-VP62 isolate (GenBank: EF185282) was from NIH (NIH 11881, [21]). The IN-encoding gene was PCR amplified with oligonucleotides XMRVIN1 and XMRVIN2 (Table S1), the first oligonucleotide introducing an *Nde*I site at the start codon and the second oligonucleotide adding a *Bam*HI site after the stop codon. The PCR product was cloned into the pGEM-T-easy vector (Promega, France) and its sequence verified (MWG biotech, France). The *Nde*I-*Bam*HI fragment was subcloned into the pET15b vector (Novagen) leading to the addition of a His tag site at the N-terminus of the protein.

2.3. Protein production, purification and Western-blot analysis

Production and purification were performed essentially as previously described [20]. First, *Escherichia coli* BL21(DE3) RILP bacteria were transformed with the pET15b-IN-XMRV plasmid. A culture of 500 ml was grown to an OD_{600nm} = 0.8, induced by addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 3 h at 37 °C. The cells were pelleted by centrifugation at 4000 *g* for 30 min and resuspended in buffer A (50 mM Tris–HCl pH 8, 1 M NaCl, 4 mM β-mercaptoethanol). Of note, for cross-linking experiments, Tris buffer was replaced by non-amine HEPES buffer. After cell disruption using a French pressure cell press and centrifugation at 10,000 *g* for 30 min, the supernatant was filtered over a 0.45 μm filter and incubated with 4 ml Ni-NTA agarose (Qiagen) overnight at room temperature. Washes with buffer A, and buffer A supplemented with 80 mM imidazole, followed by elution with buffer A supplemented with 1 M imidazole and 20 μM $ZnSO_4$ were carried out at room temperature. IN stock preparations (~14 μM) were stored at −80 °C in storage buffer (20 mM Tris–HCl pH 8, 50 μM $ZnSO_4$, 1 M NaCl, 4 mM β-mercaptoethanol and 10% glycerol). Six μg of purified IN were separated by SDS-PAGE and stained with Coomassie reagent (Instant blue, Gentaur, France). The proteins were blotted onto a PVDF transfer membrane, subjected to standard Western blot analysis using anti-6-His antibody (Qiagen, France), and detected using an ECL-plus detection kit (GE Healthcare, France) according to the manufacturer's recommendations.

2.4. Time resolved fluorescence measurements

The method has been previously described [22,23]: 400 nM IN were diluted in 20 mM HEPES pH 7, 5 mM dithiothreitol (DTT) and 10 mM $MgCl_2$, and the fluorescence of tryptophan was recorded for