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Engineering a switch-on peptide to ricin A chain for increasing its specificity towards HIV-infected cells

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ABSTRACT

Background: Ricin is a type II ribosome-inactivating protein (RIP) that potently inactivates eukaryotic ribosomes by removing a specific adenine residue at the conserved α-sarcin/ricin loop of 28S ribosomal RNA (rRNA). Here, we try to increase the specificity of the enzymatically active ricin A chain (RTA) towards human immunodeficiency virus type 1 (HIV-1) by adding a loop with HIV protease recognition site to RTA.
Methods: HIV-specific RTA variants were constructed by inserting a peptide with HIV-protease recognition site either internally or at the C-terminal region of wild type RTA. Cleavability of variants by viral protease was tested *in vitro* and in HIV-infected cells. The production of viral p24 antigen and syncytium in the presence of C-terminal variants was measured to examine the anti-HIV activities of the variants.
Results: C-terminal RTA variants were specifically cleaved by HIV-1 protease both *in vitro* and in HIV-infected cells. Upon proteolysis, the processed variants showed enhanced antiviral effect with low cytotoxicity towards uninfected cells.
Conclusions: RTA variants with HIV protease recognition sequence engineered at the C-terminus were cleaved and the products mediated specific inhibitory effect towards HIV replication.
General significance: Current cocktail treatment of HIV infection fails to eradicate the virus from patients. Here we illustrate the feasibility of targeting an RIP towards HIV-infected cells by incorporation of HIV protease cleavage sequence. This approach may be generalized to other RIPs and is promising in drug design for combating HIV.

1. Introduction

Ribosome-inactivating proteins (RIPs) are *N*-glycosidases that cleave a specific adenine on 23S or 28S ribosomal RNA (rRNA) and inactivate the ribosome for protein synthesis. Many RIPs such as trichosanthin (TCS), pokeweed antiviral protein (PAP), *Momordica* anti-HIV protein 30 (MAP30) and saporin have been found to have anti-viral activity against a board spectrum of viruses, including tobacco mosaic virus (TMV), herpes simplex virus (HSV), human immunodeficiency virus (HIV) and poliovirus [1–7].

It has been found that RIPs are capable of interfering various steps of viral cycle, including reverse transcription [8], synthesis of viral DNA [9] and integration into host chromosome [2,10], or acting on viral RNA directly [11,12]. These in turn inhibit viral replication.

A number of studies have examined the use of RIPs to inhibit HIV. Saporin and luffin strongly inhibit HIV-1 integrase [2] while several RIPs such as marmorin, hypsin and velutin have been shown to inhibit reverse transcriptase [8,13,14]. TCS and PAP have been put for clinical trials for treating AIDS patients. TCS has been found to alleviate the loss of CD4+ cells and reduce serum p24 antigen production in AIDS patients [15–17]. Immunotoxin of PAP coupled with antibodies against CD4, CD5 or CD7 also lowers the viral p24 level in AIDS patients but the immunotoxin elicits antibodies against both the toxin and CD7 [18].

Our group has recently solved the structure of maize RIP [19]. The precursor (Pro-RIP) contains a 25aa internal loop that presumably obstructs the docking of the protein to the ribosomal P protein and proteolysis is needed to remove the loop for full function [20]. We have subsequently inserted the HIV-1 protease recognition sites to the inactivation loop and found that the engineered toxin is activated by viral protease in HIV-infected cells and mediates anti-HIV effect [21]. However, most RIPs lack the natural internal loop for regulating enzymatic activity. We therefore set forth to find if this 'switch-on' mechanism can be applied to other RIPs to attain controlled protein activation. In this study, we have selected a potent RIP, ricin A chain (RTA), as test protein and constructed various HIV sensitive variants by incorporating the HIV-1 protease cleavage sequence within RTA for inhibitory obstruction. The RTA variants are tested for specific cleavage by viral protease and then anti-HIV activity.

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2. Materials and methods

2.1. Design of HIV-1 specific RTA variants

To achieve HIV-1 targeting, recognition sequence of HIV-1 protease at matrix/capsid (MA/CA) site in the polyprotein precursor Gag of HIV-1 was inserted within or at the C-terminal region of RTA (Fig. 1A). MA/CA site chosen in this study has high k_{cat}/K_m value [22,23] and is cleaved at the early stage of HIV-1 protease processing [24]. One or two MA/CA sites were inserted at the middle or C-terminal region of RTA. For the internal insertion variants RTA-10A and RTA-10B (upper panel; Fig. 1B), one cleavage sequence was introduced. Three amino acids were deleted in RTA-10B to minimize the change in length resulted from sequence insertion. RTA-25 contained two MA/CA recognition sequences spaced by a pentapeptide MQMPE. For the C-terminal variants (lower panel; Fig. 1B), RTA-C10 was engineered with one MA/CA site and RTA-C25 with two MA/CA sites at the C-terminus of RTA. RTA-C10V and RTA-C25V were two corresponding non-cleavable counterparts.

2.2. Cloning, expression and purification of RTA variants

Insertion of nucleotides to the DNA of wild type RTA was carried out by PCR using Phusion DNA polymerase (Finnzymes) and primers containing the desired modifications (Table 1). DNA of RTA with C-terminal modified was cloned into expression vector pET28a and those with an additional internal sequence were cloned into Mini-pRSETA-SUMO vector. The modified DNA was sequenced to confirm correct mutagenesis. All variants except RTA-25 were expressed in LB using BL21(DE3)pLysS at 25 °C for 5 h after induction by 0.4 mM IPTG. RTA-25 was expressed in auto-induction medium using BL21(DE3)pLysS at 16 °C for over 16 h. Then, cells were sonicated in 20 mM phosphate buffer, 800 mM NaCl, 50 mM imidazole, pH 7.4 and soluble lysate was loaded to a 5 ml HisTrap High Performance column (GE Healthcare) for affinity purification. Protein was then eluted using 20 mM phosphate buffer, 300 mM NaCl, 300 mM imidazole, pH 7.4. His-tag of RTA-WT was removed by thrombin (Sigma) and size-exclusion chromatography using Superdex 200. His–SUMO tag of

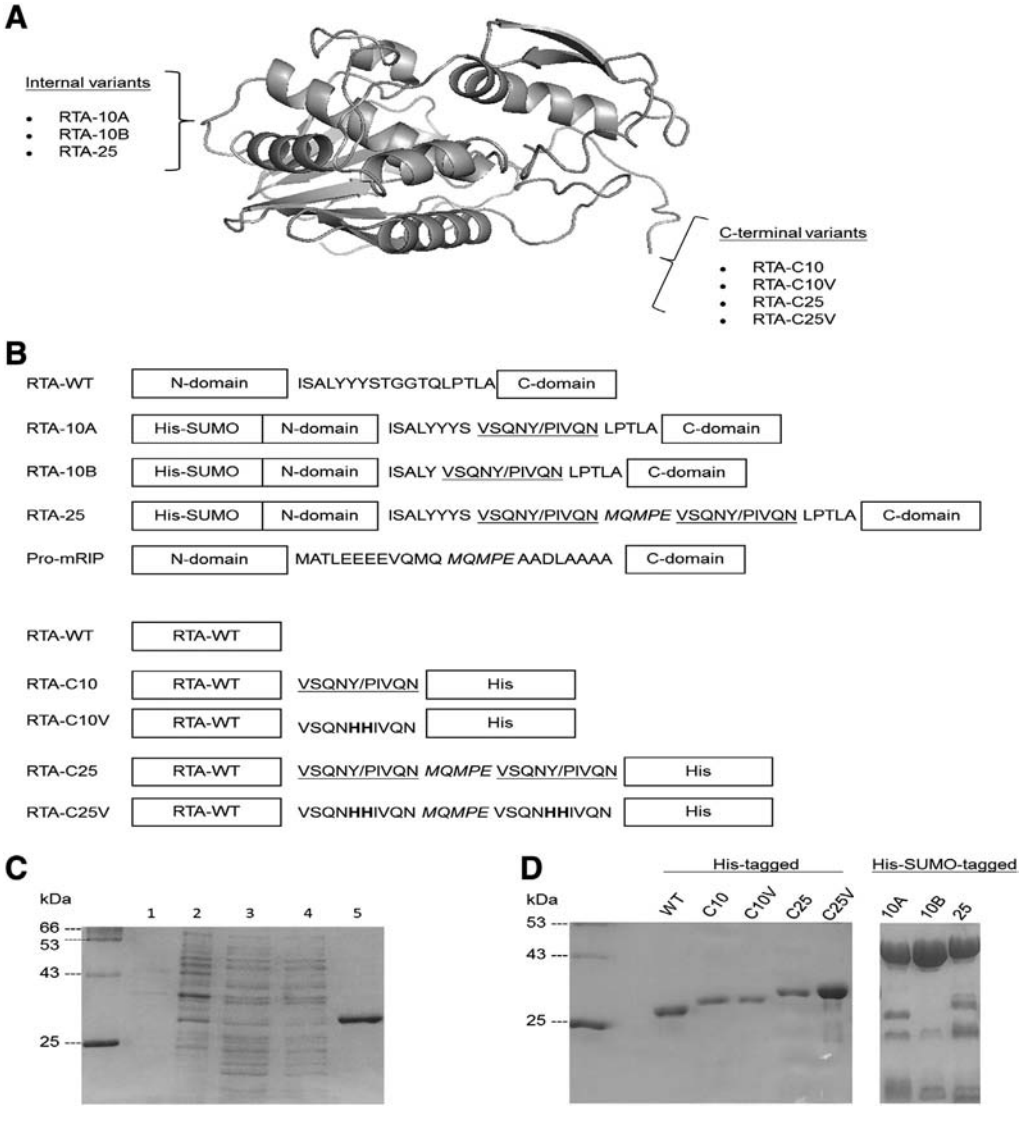

Fig. 1. Design and preparation of RTA variants. (A) Crystal structure of ricin A chain (pdb: 1RTC). HIV-1 recognition sequences were inserted at the middle or C-terminal region of RTA to generate internal and C-terminal variants. (B) Schematic diagram of RTA variants. Upper panel: HIV-1 protease recognition sequence (underlined) was inserted within RTA. RTA-10A and RTA-10B contain one cleavage site but three amino acids were removed in 10B to minimize the change in length due to sequence insertion. RTA-25 contains two HIV-1 specific sequences separated by MQMPE (italic) which is the middle five residues of Pro-mRIP insertion. Pro-mRIP is the precursor of maize RIP with a 25aa internal inactivation loop. Lower panel: RTA-C10 and RTA-C25 were generated by inserting one and two HIV-1 specific sequences respectively at C-terminus of RTA in prior to His-tag. RTA-C10V and RTA-C25V are the corresponding non-cleavable counterparts with two middle residues of sequence modified to HH (bolded). (C) Affinity purification of RTA variants exemplified by RTA-C10 (32.1 kDa). Lane 1: cell lysate before induction; lane 2: cell lysate after induction; lane 3: soluble fraction; lane 4: flow-through of Ni-affinity column; lane 5: purified RTA-C10 from Ni-column. (D) SDS-PAGE analysis of C-terminal (His-tagged) and internal (His–SUMO-tagged) RTA variants isolated by affinity purification.