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

Ka-Yee Au [a,1], Rui-Rui Wang [b,1], Yuen-Ting Wong [a], Kam-Bo Wong [a], Yong-Tang Zheng [b,*], Pang-Chui Shaw [a,**]

[a] Biochemistry Programme and Centre for Protein Science and Crystallography, School of Life Sciences, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China
[b] Key Laboratory of Animal Models and Human Disease Mechanisms, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, China

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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.

* Correspondence to: Y.-T. Zheng, Key Laboratory of Animal Models and Human Disease Mechanisms, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, China. Tel.: +86 871 65195684.
** Correspondence to: P.-C. Shaw, School of Life Sciences, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China. Tel.: +852 39431363.
E-mail addresses: zhengyt@mail.kiz.ac.cn (Y.-T. Zheng), pcshaw@cuhk.edu.hk (P.-C. Shaw).
[1] Equal contribution.

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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

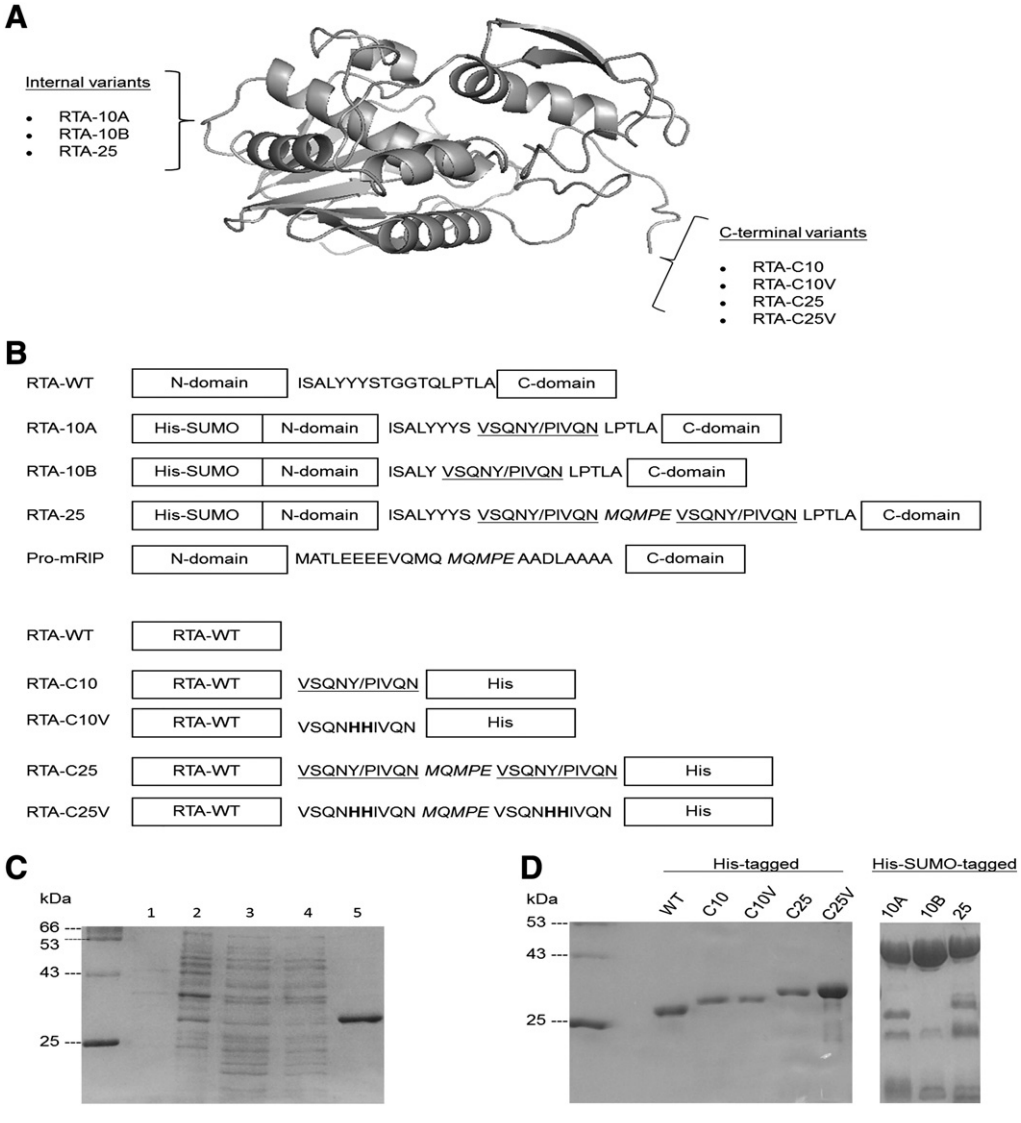

Fig. 1. Design and preparation of RTA variants. (A) Crystal structure of ricin A chain (pdb: 1RTC). HIV-1 recognition sequences were inserted at the middle or C-terminal region of RTA to generate internal and C-terminal variants. (B) Schematic diagram of RTA variants. Upper panel: HIV-1 protease recognition 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.

Table 1
List of primers used to construct the RTA variants.

Primer	Sequence (5′-3′)
RTA-NcoI-F	ATACCATGGGAGATATTCCCCAAACAATACCCAATTATAAACTTTACCACAGC
C-His-RTA-EcoRI-R	CGTTGAATTCGCAAACTGTGACGATGGTGGAGG
RTA-PstI-F	CGATATCTGCAGCATATTCCCCAAACAATACCC
RTA-EcoRI-R	CGTTGAATTCTCAAAACTGTGACGATGGTGGAGG
RTA-HIV-10A-F/ RTA-HIV-10B-F	CAAAATTATCCTATTGTTCAAAATCTTCCAACTCTGGCT
RTA-HIV-10A-R	AACAATAGGATAATTTTGACTAACACTGTAATAATAAAG
RTA-HIV-10B-R	AACAATAGGATAATTTTGACTAACATAAAGCGCTGAGAT
RTA-HIV-25-F	ATGCAAATGCCAGAGGTATCCCAGAACTACCCCATCGTACAGAAACCTTCCAACTCTGGCT
RTA-HIV-25-R	TACCTCTGGCATTTGCATATTTTGAACAATAGGATAATTTTGACTAACACTGTAATAATAAAG
RTA-C10-XhoI-28a-R	CGTTGGCTCGAGATTTTGAACAATAGGATAATTTTG
RTA-C25-XhoI-28a-R	CGTTGGCTCGAGATTCTGTACGATGGGGTAGTTCTGGGATACCTC
RTA-C10-var-XhoI_R	CGTTGGCTCGAGATTTTGAACAATATGATGATTTTGACTAACAAACTGTGACGATGGTGG
RTA-C25-var-XhoI_R	CGTTGGCTCGAGATTCTGTACGATATGATGGTTCTGGGATACCTC

the internal variants was first removed by His–SUMO protease and the mixture was loaded to a 5 ml HisTrap column for removing the tag and protease. All variants had the buffer exchanged to 20 mM phosphate buffer, 200 mM NaCl, 10% glycerol, pH 7.4 for storage at −80 °C.

2.3. Expression and purification of HIV-1 protease

Plasmid encoding the cDNA of HIV-1 protease was provided by Prof. C.C. Wan of the Chinese University of Hong Kong. The cDNA was cloned into expression vector pET3b and expressed in BL21 (DE3)pLysS at 37 °C for 4 h under the induction of 0.4 mM IPTG. The protein was expressed as inclusion bodies and purified as described previously [25]. In brief, cells were sonicated in 10 mM Tris, 2 mM EDTA, pH 8.0 (buffer A) and the lysate was centrifuged at 10,000 *g* for 10 min. The pellet was then resuspended in buffer A with Triton X-100 for repeated centrifugation. The resultant pellet was dissolved in 10 mM Tris, 8 M urea, 10 mM DTT, pH 7.5 and centrifuged at 100,000 *g*. The supernatant was then loaded to a 5 ml DEAE column (GE Healthcare) and the flow-through containing HIV-1 protease was collected and dialyzed against 10 mM sodium acetate, 1 mM DTT, 1% glycerol, pH 3.5 for refolding. The refolded protein was concentrated and had the pH adjusted to 4.4 with 100 mM sodium acetate, 1 M NaCl, pH 4.4. After centrifugation, the supernatant was collected for dialysis against 10 mM sodium acetate, pH 3.5 and concentrated for storage.

2.4. Cells and virus

Human T lymphocyte cell line (C8166) and the laboratory-derived virus (HIV-1_{IIIB}) were obtained from AIDS Reagent Project, Medical Research Council, UK. C8166 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Invitrogen) and cultured at 37 °C in a humidified incubator with 5% CO_2. The cells used in all experiments were in log-phase growth. HIV-1_{IIIB} was propagated in human T lymphocyte H9 cell line. The 50% HIV-1 tissue culture infection dose ($TCID_{50}$) in C8166 cells was determined by the Reed and Muench method [26]. The titer of the virus stock was 3.06×10^7 $TCID_{50}$ per milliliter. Virus stocks were stored in aliquots at −70 °C.

2.5. Cleavage of RTA variants by HIV-1 protease in vitro and in HIV-1 infected C8166 cells

To show cleavage of RTA variants by HIV-1 protease *in vitro*, RTA variants were incubated with viral protease in 50 mM sodium acetate, 200 mM NaCl, 10% glycerol, pH 5.5 at 1:1 or 2:1 ratio for 6 h at 37 °C. The reaction was stopped by adding 5× protein dye and then analyzed by SDS-PAGE. To show cleavage of the RTA variants in HIV-infected cells, C8166 cells were seeded on 6-well plate at density of 1×10^6 cells/well and infected with HIV-1 at multiplicity of infection (M.O.I.) of 0.75. Cells were incubated with 5 μM of C-terminal variants for 72 h at 37 °C. Then, cells were washed with PBS and lysed by addition of 5× protein dye and heated at 95 °C for 10 min. Supernatants were analyzed by Western blot and probed with polyclonal rabbit anti-ricin A chain (Abcam) and anti-rabbit (Invitrogen) antibodies. β-actin was also detected as loading control and probed by monoclonal mouse anti-actin (GenScript) and anti-mouse (Rio-Rad) antibodies.

2.6. Cytotoxicity assay

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. C8166 cells were seeded at density of 4×10^4 cell/well on 96-well plate and treated by different amounts of proteins. After incubated at 37 °C for 72 h, cells were exposed to MTT (5 mg/ml) for 4 h. The purple formazan was dissolved in 50% dimethylformamide and 20% SDS and $OD_{570/630}$ values were measured. The results were presented as mean ± standard deviation (SD) and differences among groups were analyzed by one-way analysis of variance using the software GraphPad Prism 5. Protein concentration that inhibited cell survival for 50% (CC_{50}) was determined.

2.7. Syncytium reduction assay

C8166 cells were seeded at density of 4×10^4 cells/well on 96-well plate and inoculated with HIV-1 at M.O.I. of 0.075. RTA variants were serially diluted in cell culture medium and added to cells. Infected cells incubated without protein were used as control. After incubated at 37 °C for 72 h in 5% CO_2, the number of syncytia in each well was counted under microscope and the inhibitory percentage of syncytium formation was determined by comparing the number of syncytia in treated sample with that of the control. One-way analysis of variance was performed using the software GraphPad Prism 5. Dose–response curves were plotted and the concentration of protein that inhibited syncytium formation for 50% (EC_{50}) was determined.

2.8. Viral p24 antigen reduction assay

C8166 cells were treated with RTA variants as mentioned in syncytium reduction assay and culture supernatants were collected and treated with Triton X-100. The level of viral p24 antigen in culture supernatant was measured by ELISA as described previously [27]. Percentage inhibition of p24 antigen production was determined by comparing the readings of the tested sample with control. Results were statistically analyzed by one-way analysis of variance.

3. Results

3.1. Purification of internal and C-terminal modified RTA

Recognition sequence of HIV-1 protease at MA/CA site was inserted at the middle or C-terminal region of RTA (Fig. 1B) to sensitize the protein towards HIV-1 protease. Both the internal and C-terminal variants carried His-tag for affinity purification. As exemplified by RTA-C10, the engineered protein was retained in column by His–nickel interaction (lane 4; Fig. 1C) and by eluting with high imidazole, pure protein was obtained in the eluted sample (lane 5; Fig. 1C). C-terminal variants were isolated in high purity whereas the internal variants were about 90% pure (Fig. 1D). The internal variants were prone to precipitation after the removal of His–SUMO tag and could not be enriched to sufficient soluble concentration for subsequent culture assays.

3.2. Cleavage of RTA variants in vitro and in HIV-1 infected cells

The C-terminal variants RTA-C10 and RTA-C25 were subjected to further analyses. HIV-1 protease was purified (Fig. 2A) and shown to cleave the RTA variants completely *in vitro* (Fig. 2B). The change of two residues within MA/CA site caused RTA-C10V and RTA-C25V non-cleavable (Fig. 2B). Cleavability of C-terminal variants by endogenous HIV-1 protease was also compared in HIV-1_{IIIB} infected cells. Western blot analysis showed that the cleavable RTA-C10 and RTA-C25 were cleaved to large extent and non-cleavable RTA-C10V and RTA-C25V remained intact (Fig. 2C). RTA-WT was not cleaved as expected.

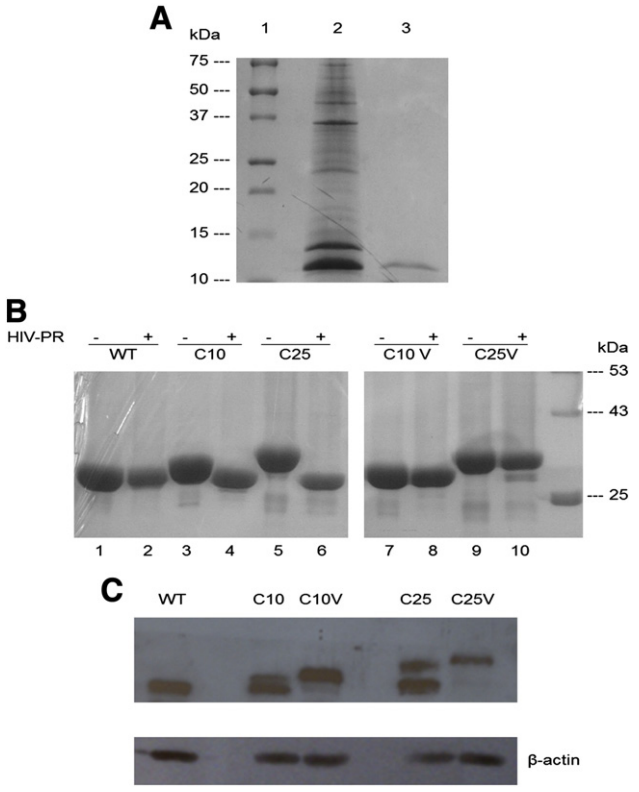

Fig. 2. Cleavage of C-terminal RTA variants. (A) Purification of HIV-1 protease (10.8 kDa). Lane 1: protein molecular weight marker; lane 2: cell lysate after induction; lane 3: purified HIV protease. (B) *In vitro* digestion of His-tagged C-terminal variants by HIV-1 protease. (C) Cleavage of C-terminal variants in HIV-1_{IIIB} acutely infected C8166 cells. C10 and C25 were partially cleaved and detected with size similar as WT while non-cleavable counterparts remained intact.

Table 2
Cytotoxicity and antiviral activity of C-terminal variants in uninfected and HIV-1_{IIIB} acutely infected C8166 cells. The values are presented as means ± SD (n = 6).

	Uninfected	HIV-1_{IIIB}-infected	
	Cytotoxicity CC_{50} (μM)	P24 antigen reduction EC_{50} (μM)	Syncytial reduction EC_{50} (μM)
RTA-WT	6.56 ± 0.75	0.46 ± 0.02	0.51 ± 0.05
RTA-C10	>10	1.27 ± 0.11	4.03 ± 0.09
RTA-C10V	>10	3.63 ± 0.44	7.09 ± 0.07
RTA-C25	>10	0.97 ± 0.10	3.82 ± 0.24
RTA-C25V	>10	3.09 ± 0.15	6.11 ± 0.08

3.3. Anti-HIV activity of C-terminal RTA variants

The cytotoxic effect of RTA variants on uninfected C8166 cells and inhibition of HIV replication on HIV-1_{IIIB} infected C8166 cells were examined. For RTA-WT, the cytotoxic and anti-HIV activity displayed a positive correlation with concentration. The syncytium formation and viral p24 antigen production were inhibited for 50% at 0.51 ± 0.05 and 0.46 ± 0.02 μM respectively with 50% cell viability at 6.56 ± 0.75 μM (Table 2). All RTA variants tested were less cytotoxic than the RTA-WT. The HIV-1 sensitive RTA-C10 and RTA-C25 showed comparable inhibitory effects on p24 antigen production as the wild type but were less efficient on syncytial reduction. Compared to their non-cleavage counterparts RTA-C10V and RTA-C25V, the cleavable variants were more potent in inhibiting the generation of p24 antigen and syncytia (Table 2).

4. Discussion

Previously, our group has converted maize RIP, a type III RIP with a naturally occurring internal inactivation loop, into an anti-HIV agent [21]. The internal loop in maize RIP precursor suppresses ribosome-inactivating activity by more than 6600-fold [28] as it masks the protein for getting access to the ribosome [20]. We increased the specificity of maize RIP towards HIV-1 by engineering HIV-1 recognition sites to the internal loop.

The corresponding inactivation loop is absent in type I and II RIPs. Previously, we have shown that trichosanthin (TCS) with an additional 19aa at C-terminus is about five times less active than the mature protein [29]. Therefore, we use RTA as a test case and insert HIV-1 specific sites either within the protein to mimic the inactivation loop of maize RIP or at C-terminal region to show the feasibility of the switch-on loop approach.

RIPs are highly allergenic attributed to their botanical origin and administration of RIP products often leads to strong immune responses [30–32]. Computer modeling of ricin A chain was performed to predict potential antibody epitopes and suggested the corresponding internal and C-terminal insertion sites of HIV-1 protease sequences were of high antigenic index (not shown). Thus the RTA variants may also have alleviated allergenicity compared to the wild-type ricin as the insertions may mask the epitopes for immune responses.

The cleavable C-terminal variants RTA-C10 and RTA-C25 were specifically cut by HIV-1 protease *in vitro* and in HIV-infected cells whereas their non-cleavable counterparts remained intact as expected (Fig. 2). Proteolytic action of the viral protease is of high specificity [33] and substitution of two amino acids within recognition sequence added in RTA-C10V and RTA-C25V is sufficient to abolish the cleavage.

On the other hand, the engineering of internal RTA variants was less fruitful as they precipitated and could not be enriched to concentration required for culture assays. Although the switch-on peptide is inserted at an exposed loop of RTA at the same location of maize RIP and the peptide is supposed to stay on the surface of RTA, this insertion or the charges on the peptide may affect the proper folding of RTA.

The C-terminal RTA variants were further tested in HIV-infected cells for anti-HIV activity. Compared to the wild type, HIV-protease sensitive variants RTA-C10 and RTA-C25 were less toxic than RTA for uninfected cells. They were more active to HIV-1 infected cells compared to the HIV-protease insensitive variants (Table 2). Compared to wild-type RTA, the HIV-protease sensitive variants were less potent in inhibiting syncytial formation. Syncytial formation necessitates the interaction between CD4 receptor on uninfected cells and gp120 envelope protein on HIV-infected cells [34,35]. Affinity of gp120 for CD4 directly correlates with the syncytium forming efficiency [36]. This process appears to be independent of p24 expression as similar p24 antigen level was reported in infections by syncytium-inducing and non-syncytium inducing HIV [37,38].

The HIV-1 protease recognition sequences have been engineered into various cytotoxic proteins to generate anti-HIV agents. Falnes and co-workers linked diphtheria toxin and N-end-rule-mediated degradation signals with HIV-1 recognition sites and found that the fusion protein was exclusively cleaved at designated site. The processed protein was stabilized and exhibited increased inhibition on cellular protein synthesis but cleavage in HIV-infected cells could not be detected [39]. Two groups separately joined the N- and C-termini of ribonuclease A with HIV-1 specific sequence to form a circular permutation for obstructing the active site. *In vitro* cleavage by HIV protease was shown in both studies and the cleaved proteins had the ribonucleolytic activity increased [40,41]. Vocero-Akbani and co-workers worked on the apoptotic caspase-3 which is proteolytically activated by upstream caspases. They replaced the cleavage site in caspase-3 with HIV-1 recognition sequence. The engineered protein was cut in Jurkat T cells transduced with HIV-1 protease and mediated apoptosis [42]. Our work on HIV specific RTA variants demonstrated that the C-terminal modified proteins could be efficiently cleaved by both recombinant and endogenous HIV protease. In HIV-infected cells, the processed variants exhibited desirable anti-HIV activity by reducing the production of p24 antigen and syncytium. Our work suggests that the C-terminal region of RTA can act as the activity control site. This approach may also be extended to other RIPs without internal inactivation loop, as the precursors of many of them have additional amino acids in their C-termini.

Currently, HIV-infected patients are administered with a combination of three or more drugs for inhibiting at least two viral replication stages, commonly known as Highly Active Antiretroviral Therapy (HAART). However, it fails to eradicate HIV as isolation of viral RNA from plasma of HAART-treated patients [43] indicates the continuation of viral replication. The variants generated by our strategy may be further explored as a complementary tool for treating AIDS patients. The positive results on RTA engineering also imply that the C-terminal region of RIPs can serve as regulatory site to achieve specific protein activation.

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