Toxins that are activated by HIV type-1 protease through removal of a signal for degradation by the N-end-rule pathway

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Diphtheria toxin enters the cytosol of mammalian cells where it inhibits cellular protein synthesis, leading to cell death. Recently we found that the addition of a signal for N-end-rule-mediated protein degradation to diphtheria toxin substantially reduced its intracellular stability and toxicity. These results prompted us to construct a toxin containing a degradation signal that is removable through the action of a viral protease. In principle, such a toxin would be preferentially stabilized, and thus activated, in cells expressing the viral protease in the cytosol, i.e. virus-infected cells, thereby providing a specific eradication of these cells. In the present work we describe the construction of toxins that contain a signal for N-end-rule-mediated degradation just upstream of a cleavage site for the protease from HIV type 1 (HIV-1 PR). We show that the toxins are cleaved by HIV-1 PR exclusively at the introduced sites, and thereby are converted from unstable to stable proteins. Furthermore, this cleavage substantially increased the ability of the toxins to inhibit cellular protein synthesis. However, the toxins were unable to selectively eradicate HIV-1-infected cells, apparently due to low cytosolic HIV-1 PR activity, since we could not detect cleavage of the toxins by HIV-1 PR in infected cells. Alternative strategies for the construction of toxins that can specifically be activated by viral proteases are discussed.

Key words: anthrax toxin, diphtheria toxin, lethal factor.

INTRODUCTION

Several plant and bacterial toxins act on intracellular targets in an enzymic fashion, leading to perturbation of cellular physiology or to cell death. Such toxins consist generally of two functionally distinct structures, A and B. The A moiety is an enzyme that is translocated to the cytosol, where it exerts its action, whereas the B moiety provides binding of the toxin to the cell surface, and in some cases also forms a pore that facilitates the translocation of the A moiety to the cytosol.

Diphtheria toxin is synthesized as one polypeptide chain that is split by low concentrations of trypsin into its A (21 kDa) and B (37 kDa) moieties (also denoted the A- and B-fragments), joined by a disulphide bond. Upon binding to cell-surface receptors, the toxin is endocytosed, and the acidic pH in the endosome triggers unfolding of the toxin, leading to translocation of the A-fragment to the cytosol. Also, if cell-surface-bound toxin is exposed to low pH, thus mimicking the conditions in the endosome, a direct translocation of the A-fragment to the cytosol is induced (see [1] for a review on diphtheria-toxin entry).

The anthrax toxins consist of two different A moieties, denoted lethal factor (LF) and oedema factor, that in a mutually exclusive fashion can associate with a common B moiety, the protective antigen (PA), thereby forming the lethal toxin and the oedema toxin, respectively. Oedema factor is a calmodulin-dependent adenylate cyclase [2], whereas LF is a protease that cleaves mitogen-activated protein kinase kinase [3]. Upon binding of PA (83 kDa) to the cell receptor, a 20-kDa fragment is cleaved off by a cell-surface protease, probably furin, leading to exposure of the binding site for LF and oedema factor [4]. This cleavage, also referred to as 'nicking', can also be obtained when PA is treated with low concentrations of trypsin *in vitro*. As in the case of diphtheria toxin, the translocation of the anthrax toxin A moieties to the cytosol is triggered by the low pH in the endosome, and a direct translocation across the plasma membrane is induced when surface-bound toxin is exposed to acidic medium [5]. Amino acids 1–255 of LF are denoted LF_N, and encompass the PA-binding region of LF. Previous studies have shown that when the A moieties of various other protein toxins, including diphtheria toxin, are fused to LF_N, the resulting fusion proteins can be translocated to the cytosol in a PA-dependent fashion [6].

During the infection of a cell by a virus, the generation of new viral particles commonly involves the cleavage of precursor proteins by a specific, virally encoded, protease (reviewed in [7]). In the case of HIV, the structural components matrix protein, capsid protein (CA) and nucleocapsid protein are generated through cleavage of the Gag polyprotein by HIV type-1 protease (HIV-1 PR). The Gag-Pol polyprotein is formed by translational frameshifting in the 3′ region of the *Gag* gene, and HIV-1 PR itself is excised autocatalytically from this precursor. Virus replication depends on the activity of HIV-1 PR, and the development of specific inhibitors of HIV-1 PR has led to improved treatment of AIDS. No true consensus sequence for the substrate specificity of HIV-1 PR exists, but the cleavage occurs at defined specific sites. The processing of HIV-1 polyproteins by HIV-1 PR is thought to be coupled to the budding of viral particles, and to occur primarily in the viral particles before or immediately after budding. However, it has also been reported that a substantial degree of polyprotein processing by HIV-1 PR can take place in the cytosol of acutely infected cells [8], and that some cytosolic proteins are subject to cleavage by HIV-1 PR in acutely infected cells [9–11].

The N-end rule for protein degradation (reviewed in [12]) relates the intracellular half-life of a protein to its N-terminal residue, and it was initially discovered in yeast. The N-end-rule pathway was later described in bacteria and mammalian cells, and prototype destabilizing residues in mammalian cells are bulky hydrophobic amino acids (Phe, Trp, His, Tyr) and charged

Abbreviations used: HIV-1 PR, HIV type-1 protease; LF, lethal factor; LF_N, amino acids 1–255 of LF; PA, protective antigen; CA, capsid protein; DTA, diphtheria toxin A-fragment; TCA, trichloroacetic acid; FLD, LF_N–DTA preceded by a FLAG peptide containing an N-terminal Phe residue.

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amino acids (Glu, Asp, Arg, Lys, His). We found previously that the toxicity of diphtheria toxin could be modulated through the addition of a degradation signal for N-end-rule-mediated degradation to the diphtheria toxin A-fragment (DTA) [13], and that this degradation signal also could destabilize LF_N when fused to the N-terminus of this protein [5].

Varshavsky suggested previously the construction of a new kind of toxin where a signal that inactivates the toxin, e.g. a degradation signal, can be cleaved off by a viral protease [12]. Such toxins were denoted sitoxins (signal-regulated cleavage-mediated toxins). In theory, these toxins would be active only in virus-infected cells, and therefore be able to selectively eradicate these cells. Conceivably, the level of cytosolic HIV-1 PR in infected cells might be sufficiently high to allow efficient processing of a toxin containing a cleavage site for this protease. In the present work we describe the construction and characterization of two sitoxins based on a fusion between LF_N and DTA (LF_N–DTA), each containing a different cleavage site for HIV-1 PR immediately downstream of an N-terminal degradation signal. We have studied the ability of these sitoxins to become activated by HIV-1 PR *in vitro* and in HIV-infected cells.

EXPERIMENTAL

Buffers, media and reagents

The buffers and reagents used in this study were as follows: dialysis buffer, 140 mM NaCl/20 mM Hepes/2 mM $CaCl_2$, adjusted to pH 7.0 with NaOH; Hepes medium, bicarbonate- and serum-free Eagle's minimal essential medium buffered with Hepes to pH 7.4; lysis buffer, 0.1 M NaCl/20 mM NaH_2PO_4/10 mM EDTA/1 % Triton X-100/1 mM PMSF/1 mM *N*-ethylmaleimide (pH 7.4); MES-gluconate buffer, 140 mM NaCl/5 mM sodium gluconate/20 mM Mes, adjusted with Tris to pH 4.8 or 7.0; and PBS, 140 mM NaCl/10 mM NaH_2PO_4, pH 7.4. The plasmid pET-15b-LF_N-DTA [14] and purified PA were obtained from Dr. R. John Collier (Harvard Medical School, Boston, MA, U.S.A.). The HIV-1 PR inhibitor saquinavir (Ro31-8959) [15] was provided by Dr. Jan Konvalinka (Czech Academy of Sciences, Prague, Czech Republic).

Cell cultures

HeLa P4 cells [16] stably expressing the CCR5 (CC-chemokine receptor no. 5) co-receptor (HeLa P4/CCR5) were provided by M. Alizon (Institut Cochin de Genetique Moleculaire, Paris, France), and maintained in Dulbecco's modified minimal Eagle's medium supplemented with 5 % fetal calf serum. For degradation experiments and toxicity experiments, the cells were seeded into 24-well Costar plates (Costar, Cambridge, MA, U.S.A.) on the day preceding the experiments at densities of 8×10^4 and 5×10^4 cells/well, respectively.

Construction of plasmids

PCR was used to construct plasmids encoding the three mutant proteins. PCR was performed using pET-15b-LF_N-DTA [14] as a template. The forward primer was: 5′-GCGAATCCATATG-GCACATATCGAGGGAAGGTTTTACAAGGACGAGAT-GATAAGCTAGCAGGCGGTCATGGTGATGTA-3′ and the reverse primer was an oligonucleotide that annealed to the sequence corresponding to the C-terminal end of LF_N. The resulting PCR product was digested with *Nde*I and *Nsi*I, and cloned between the corresponding sites in pET-15b-LF_N-DTA, generating the plasmid pEAn-6, which encodes FLD (F refers to the N-terminal Phe, L to LF_N and D to DTA; see Figure 1). This plasmid contains a unique *Nhe*I site at the position corresponding to the start of LF_N. The plasmid, pEAn-9, which encodes FLD/MM (where MM refers to the amino acids flanking the cleavage site; see Figure 1a), was generated by performing a PCR using pEAn-6 as a template, the forward primer 5′-GATGATA-AGCTAGCAACAGCAACAATCATGATGCAGCGAGG-CGCCGGCGGTCATGGTGATGTAGGT-3′ and the same reverse primer that was used in the construction of pEAn-6. The PCR product was digested with *Nhe*I and *Nsi*I and cloned between the corresponding sites in pEAn-6. pEAn-10, which encoded FLD/YV (where YV refers to the amino acids flanking the cleavage site; see Figure 1a), was generated by the same strategy as that used when making pEAn-9, except that as a forward primer the following was used: 5′-GATGATAAG-CTAGCAGTAAGTCAGAATTATGTAATCGTTCAGGCC-GGCGGTCATGGTGATGTAGGT-3′. The sequences of the regions that had been generated by PCR were verified by dideoxy sequencing.

Transcription and translation *in vitro*

Plasmid DNA was linearized downstream of the encoding gene and transcription was carried out in a 20-μl reaction mixture with T7 RNA polymerase. The mRNA was precipitated with ethanol and dissolved in 10 μl of water containing 10 mM dithiothreitol and 0.1 unit/μl RNasin (Promega, Madison, WI, U.S.A.). The translation was performed for 1 h at 30 °C in micrococcal nuclease-treated rabbit reticulocyte lysate (Promega) containing 1 μM [^{35}S]methionine and 25 μM of each of the 19 other amino acids, and 5 μl of the dissolved mRNA was used per 100 μl of lysate.

SDS/PAGE

PAGE in the presence of SDS was carried out in 10 or 12 % gels by standard methods. After electrophoresis the gel was fixed for 30 min in 27 % methanol/4 % acetic acid, and then incubated for 30 min in 1 M sodium salicylate/2 % glycerol, pH 5.8. Kodak XAR-5 film was exposed to the dried gel in the absence of an intensifying screen at −80 C. In some cases Phosphor storage screens (Molecular Dynamics, Sunnyvale, CA, U.S.A.) were exposed to the gels.

Proteolytic processing of toxins

Translation mixtures containing [^{35}S]methionine-labelled toxins were dialysed overnight against dialysis buffer and then treated for 2 h at 25 °C with factor Xa (10 μg/ml). Subsequently, EDTA (2 mM) and dithiothreitol (1 mM) were added. In some cases, the mixture was incubated with 50 nM active-site-titrated purified HIV-1 PR for 30 min at 37 °C, in the absence or presence of 1 μM saquinavir (the specific inhibitor). HIV-1 PR had been purified as described in [11], except that cation-exchange chromatography was omitted and gel-permeation chromatography on Superose 12 (Pharmacia) was performed instead. Nicked PA was generated by incubating PA with 1 μg/ml trypsin for 30 min on ice, followed by the addition of soya bean trypsin inhibitor to a final concentration of 10 μg/ml.

Assay for analysing degradation of mutant toxins *in vivo*

HeLa P4/CCR5 cells were incubated with nicked PA (2×10^{-8} M) for 2 h at 4 °C, washed twice with cold PBS, and then incubated for 2 h at 4 °C with translation mixture containing [^{35}S]methionine-labelled toxins that had been generated through cleavage with factor Xa, and in some cases had been pretreated with HIV-1 PR. The cells were washed three times with PBS to

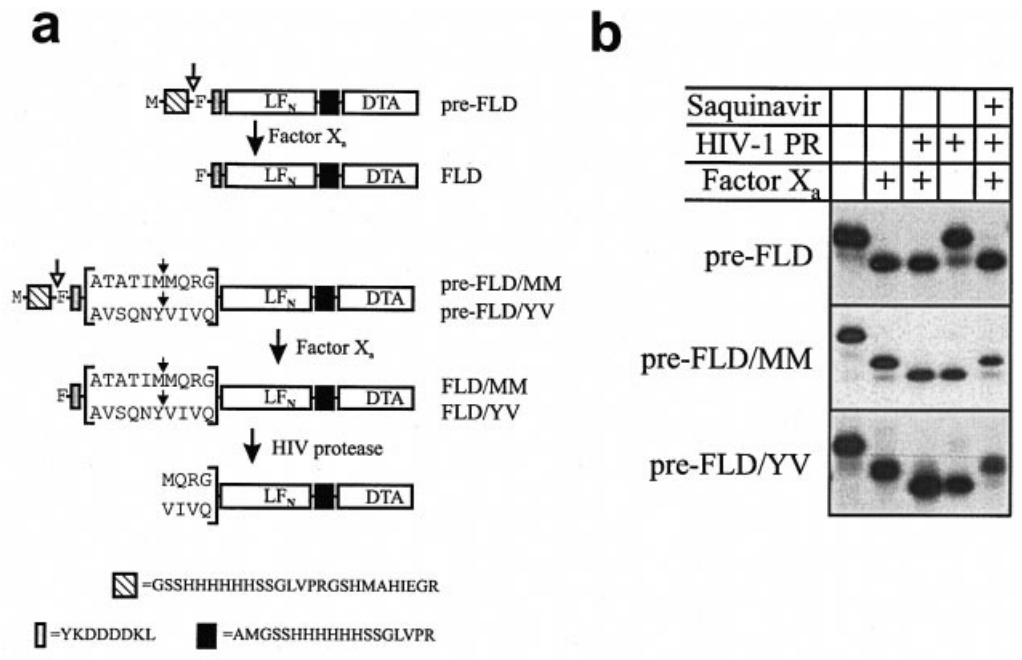

Figure 1 Generation of hybrid toxins containing cleavage sites for HIV-1 PR

(**a**) Scheme of constructs used. Each toxin was synthesized as a precursor with an N-terminal Met residue (pre-FLD, pre-FLD/MM and pre-FLD/YV). The precursor contained a recognition sequence (IEGR) for factor Xa (open arrows indicate the cleavage site), and treatment with this protease converted the toxin to a protein with an N-terminal Phe residue (FLD, FLD/MM and FLD/YV). The reported cleavage sites of the HIV-1 PR substrates TATIMMQRG and VSQNYVIVQ are indicated by small filled arrows. (**b**) Expression and proteolytic processing of hybrid toxins. [^{35}S]Methionine-labelled precursors of each toxin were generated by translation *in vitro* in a reticulocyte lysate, and, where indicated, treated with factor Xa, and subsequently incubated with HIV-1 PR in the presence or absence of the HIV protease inhibitor saquinavir. The proteins were analysed by SDS/PAGE and fluorography. To accentuate the differences in migration rate, a large (10 × 14 cm) 10% gel that was run for a long time (19 h at 100 V) was used in this experiment. For the sake of convenience and sensitivity, 12% minigels have been used in the experiments shown in subsequent Figures, and the differences in migration rate are therefore smaller.

remove unbound toxin, incubated for 2 min at 37 °C with MES-gluconate buffer, pH 7.0 or 4.8, and then incubated in Hepes medium at 37 °C for various time periods, Finally, the cells were lysed for 10 min at 0 °C in lysis buffer, trichloroacetic acid (TCA) was added to a final concentration of 5%, and the precipitated proteins were collected by centrifugation and analysed by SDS/PAGE and fluorography. Phosphor storage screens were exposed to the gels, and the intensities of the bands were quantified by using a PhosphorImager (Molecular Dynamics).

Analysis of degradation of mutant toxins *in vitro*

[^{35}S]Methionine-labelled mutant toxins were obtained by translation and cleavage with factor Xa *in vitro*. In some cases the toxins were cleaved further with HIV-1 PR. To 2 μl of [^{35}S]methionine-labelled toxin was added 13 μl of fresh reticulocyte lysate, and the mixture was incubated at 37 °C. After different time periods, 3-μl aliquots were removed, added to 27 μl of reducing SDS/PAGE sample buffer and frozen immediately. Subsequently, the samples were analysed by SDS/PAGE and fluorography. In order to prevent overloading of the gel only one third of each sample was applied to the gel.

Measurement of protein-synthesis inhibition by mutant toxins

HeLa P4/CCR5 cells were incubated for 24 h at 37 °C with 2×10^{-8} M PA and increasing concentrations of the toxins. The cells were allowed to incorporate radioactivity for 30 min at 37 °C in Hepes medium containing 2 μCi/ml [^{3}H]leucine and no unlabelled leucine. The cells were washed for 10 min with 5% TCA, washed briefly with 5% TCA, dissolved finally in 0.1 M KOH, and the cell-associated radioactivity was measured. The toxin-containing reticulocyte lysates used in the toxicity experiments were labelled with [^{35}S]methionine, but the amount of label contributed by the toxins was negligible compared with that of the incorporated [^{3}H]leucine, and therefore did not interfere with the toxicity measurements. The relative amounts of the different toxins in the reticulocyte lysates were estimated by measuring the intensities of the bands representing the toxins upon SDS/PAGE and PhosphorImaging. The actual toxin concentration was estimated in a Western blot by comparison with a dilution series of a sample of known concentration.

Measurement of specific inhibition of viral protein synthesis by mutant toxins

HeLa P4/CCR5 cells were infected with a high-titre stock of HIV-1 strain NL4-3 [17] for 4 days and seeded at a density of 5×10^4 cells/well in 24-well plates. Under these conditions 60% of the cells were infected, as determined by β-galactosidase staining *in situ* [18]. Infected cells were incubated for 16 h with PA and increasing concentrations of the toxins as described above. Radioactive labelling and immunoprecipitation of HIV-specific proteins was essentially performed as described previously [19]. Briefly, following a 1-h starvation in methionine- and cysteine-free medium with 10% dialysed fetal calf serum, cells were incubated for 1 h in the same medium containing 0.5 mCi/ml [^{35}S]methionine/cysteine (Trans-S label from ICN, specific activity > 1000 Ci/mmol). Subsequently, cells were incubated for 5 h in the presence of 0.1 mCi/ml of labelled amino

acids. PA and toxins were present in the medium throughout the procedure. Cells were lysed in 1 % SDS and lysates were adjusted to a final concentration of 20 mM Tris/HCl (pH 7.5)/150 mM NaCl/2 mM EDTA/0.5 % sodium deoxycholate/1 % Triton-X 100/0.1 % SDS. Aliquots were removed and proteins were precipitated with 5 % TCA for measurement of cell-associated radioactivity.

Immunoprecipitation of HIV Gag proteins was performed overnight using a polyclonal rabbit antiserum against HIV-1 CA adsorbed on to Protein A–agarose. The immunoprecipitates were separated by SDS/PAGE and Phosphor storage screens were exposed to dried gels. The intensities of the HIV-specific bands were quantified using a Fuji BAS 2000 bioimager.

RESULTS

Construction and processing of mutant toxins

When DTA was fused C-terminally to the first 255 amino acids of the LF of anthrax toxin (LF_N), the resulting fusion protein (LF_N–DTA) was able to inhibit cellular protein synthesis in a PA-dependent fashion [14], due to its translocation to the cytosol [5]. Previously, we found that when short sequences derived from the FLAG peptide epitope [20], differing only in their N-terminal amino acids were fused N-terminally to DTA, the intracellular stability of the resulting proteins depended on the identity of the N-terminal amino acid, i.e. they could be degraded by the N-end-rule pathway [13]. When grafted on to the N-terminus of LF_N, FLAG-like peptides with N-terminal Arg or Phe residues were also able to target this protein for degradation [5]. We have now constructed a fusion protein that consists of LF_N–DTA preceded by a FLAG peptide containing an N-terminal Phe, denoted FLD (Figure 1a). FLD was expected to be generated when a precursor, pre-FLD, which contained a recognition site for factor Xa, was treated with this protease. The plasmid encoding pre-FLD was based on a plasmid encoding LF_N–DTA with an N-terminal hexahistidine tag [14], and therefore pre-FLD also contained this tag. We also made two variants of FLD, containing two different cleavage sites for HIV-1 PR, denoted FLD/MM and FLD/YV, where MM and YV refers to the amino acids flanking the cleavage site (Figure 1a). FLD/MM contained the nonapeptide TATIM*MQRG, which represents the protease cleavage site (*) in the HIV-1 Gag polyprotein that is cleaved most rapidly, both *in vitro* and in viral maturation [21–23]. FLD/YV contains the sequence VSQNY*VIVQ, which corresponds to the cleavage site (*) between the matrix and CA domains of the HIV Gag polyprotein, but contains a Pro → Val substitution in the P1′ position of the cleavage site. This substitution was introduced in order to generate an N-terminal amino acid (i.e. Val) that was stabilizing, according to the N-end rule, upon cleavage with HIV-1 PR, since the status of Pro in the mammalian N-end rule has not been addressed *in vivo*. Also, this substitution has been shown to have only a minor effect on the cleavage of the peptide by HIV-1 PR [22,24].

Radioactively labelled precursor proteins pre-FLD, pre-FLD/MM and pre-FLD/YV were obtained by transcribing the plasmids encoding them *in vitro* from a T7 promoter, followed by translation of the mRNA in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. We then tested the ability of these proteins to be cleaved at the introduced sites for factor Xa and HIV-1 PR, and the results are shown in Figure 1(b). As expected, treatment of pre-FLD with factor Xa gave rise to an increased mobility in SDS/PAGE, but HIV-1 PR also appeared to cleave this protein very inefficiently at, or very close to, the factor Xa site. Treatment of pre-FLD/MM and pre-FLD/YV with factor Xa resulted in a mobility shift of approximately the same magnitude as that observed in the case of pre-FLD, whereas treatment with HIV-1 PR, either alone or in combination with factor Xa, led to a further increase in mobility. This indicates that the proteins were cleaved only at the introduced sites, since the site for HIV-1 PR is downstream of the factor Xa site. In the case of pre-FLD/MM, but not pre-FLD/YV, factor Xa seems to be able to cleave the protein very inefficiently at, or very close to, the HIV-1 PR site. The cleavage of FLD/MM and FLD/YV by HIV-1 PR was inhibited by the specific HIV-1 PR inhibitor, saquinavir. In summary, the three proteins appear to have been specifically cleaved by HIV-1 PR and factor Xa at the predicted sites, and only a small amount of cleavage at other sites was observed.

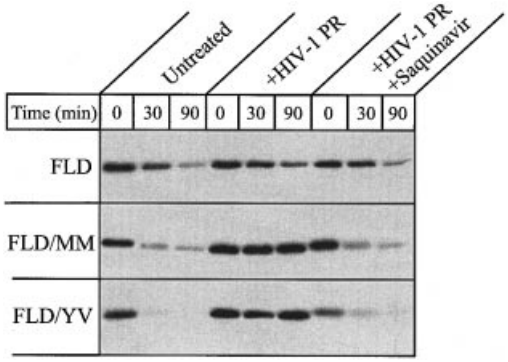

Figure 2 Stabilization of toxins *in vitro* by treatment with HIV-1 PR

FLD, FLD/MM, and FLD/YV were generated through cleavage of the respective precursors with factor Xa, and, where indicated, the proteins were treated subsequently with HIV-1 PR in the absence or presence of the inhibitor saquinavir. The proteins were then mixed with an excess of freshly thawed reticulocyte lysate and incubated at 37 °C. At the indicated time points aliquots were removed for analysis by SDS/PAGE and fluorography.

Degradation *in vitro* of toxins is severely reduced following pretreatment with HIV-1 PR

The cleavage of FLD/MM or FLD/YV with HIV-1 PR at the introduced sites would have changed the identity of the N-terminal amino acid from Phe to Met or Val, respectively. Since Phe is a destabilizing residue according to the N-end rule, whereas Met and Val are stabilizing, the treatment of the proteins with HIV-1 PR would be expected to convert them from unstable to stable proteins. N-end-rule substrates have been shown previously to be efficiently degraded *in vitro* in reticulocyte lysates [25], and we therefore investigated the stability of FLD, FLD/MM and FLD/YV in this system. Untreated FLD, FLD/MM and FLD/YV were efficiently degraded in the reticulocyte lysates, whereas treatment with HIV-1 PR resulted in a stabilization of FLD/MM and FLD/YV, but not of FLD (Figure 2). The effect of HIV-1 PR on the stability of FLD/MM and FLD/YV was not observed when the HIV-1 PR treatment was carried out in the presence of the HIV-1 PR inhibitor saquinavir. The degradation of untreated FLD/MM *in vitro* appeared to be somewhat slower than that of FLD/YV (Figure 2). However, we already observed that the treatment of pre-FLD/MM with factor Xa, in addition to generating FLD/MM, also gave a small degree of unspecific cleavage at another site, probably close to the cleavage site for HIV-1 PR, leading to a faster-migrating cleavage product (Figure 1b). When the samples from the degradation reaction shown in Figure 2 were run on a gel with higher resolution, we found that the untreated FLD/MM

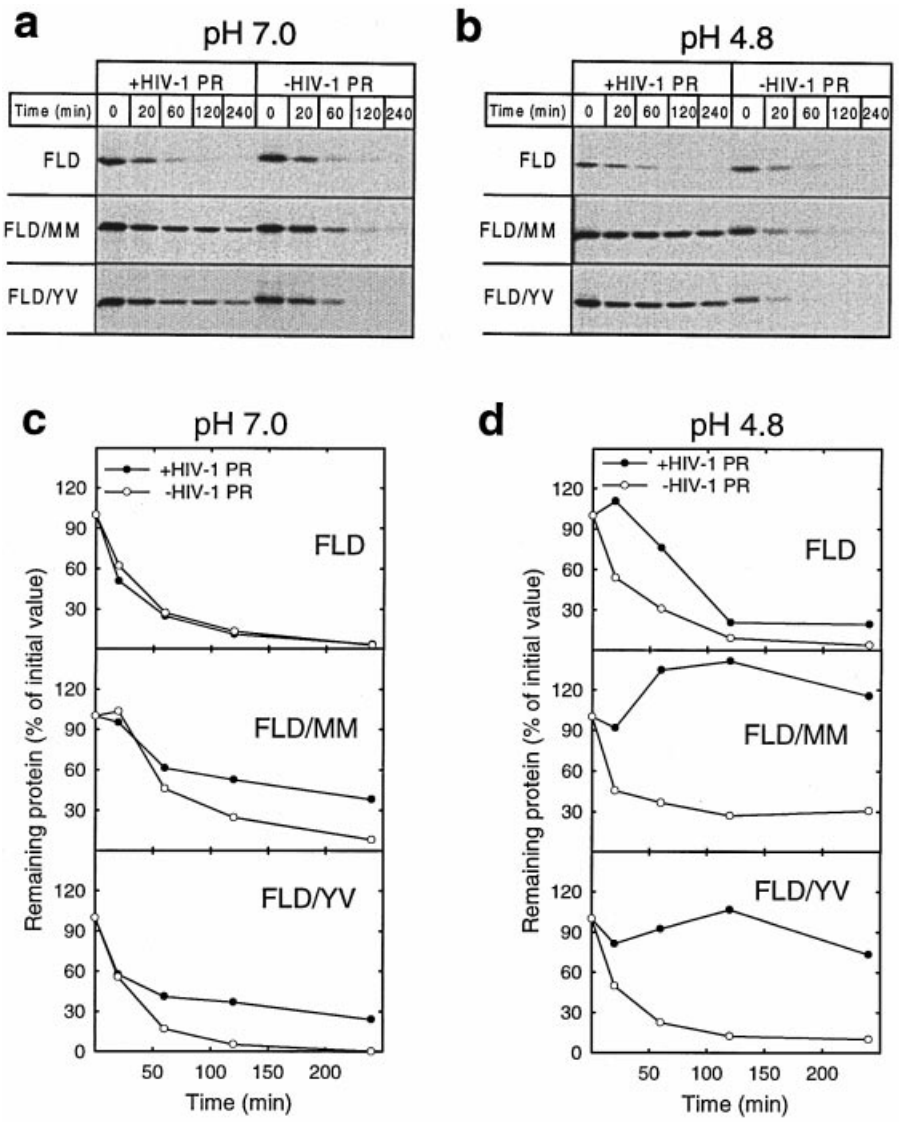

Figure 3 Stabilization of toxins *in vivo* by treatment with HIV-1 PR

HeLa P4/CCR5 cells were incubated with nicked PA (2×10^{-8} M) for 2 h at 4 °C, subsequently washed, and then incubated for 2 h at 4 °C with [^{35}S]methionine-labelled toxins that had been generated through cleavage with factor Xa, and, where indicated, had been pretreated with HIV-1 PR. The cells were then washed and incubated for 2 min at 37 °C with MES-gluconate buffer at pH 7.0 (**a**) or 4.8 (**b**), followed by incubation at 37 °C for the indicated time periods. Finally, the cells were lysed, proteins were collected by TCA-precipitation, and analysed by SDS/PAGE and fluorography. (**c**) and (**d**) Quantification by PhosphorImaging of the gels shown in (**a**) and (**b**), respectively.

remaining at the 90-min time point migrated faster than the starting material (results not shown). It is thus likely that the apparent difference in degradation rate between FLD/MM and FLD/YV does not reflect a true difference in stability between the two proteins, but rather is caused by FLD/MM being contaminated by a small amount of a stable protein generated by unspecific cleavage during the factor-Xa treatment. As expected, the precursor proteins pre-FLD, pre-FLD/MM and pre-FLD/YV, which all had N-terminal Met residues, were not degraded in the reticulocyte lysate (results not shown).

Stabilization of toxins *in vivo* by HIV-1 PR pretreatment

To test if HIV-1 PR pretreatment would also stabilize the toxins containing cleavage sites for HIV-1 PR *in vivo*, HeLa P4/CCR5 cells were first incubated with nicked PA at 4 °C, in order to bind PA to the anthrax-toxin receptors without endocytosis occurring. This was followed by incubation at 4 °C with [^{35}S]methionine-labelled FLD, FLD/MM or FLD/YV, thus allowing the labelled proteins to bind to cells through association with surface-bound PA. The cells were then washed and incubated at 37 °C, allowing the bound toxin to be endocytosed and translocated to the cytosol. After various time periods, the cells were lysed, and cellular proteins analysed by SDS/PAGE and fluorography (Figure 3a). The gels were also exposed to Phosphor storage screens, and the intensities of the bands quantified by PhosphorImaging (Figure 3c). The results of these experiments showed that pretreatment with HIV-1 PR substantially increased the stability of cell-associated FLD/MM and FLD/YV, whereas FLD was degraded rapidly irrespective of pretreatment with

HIV-1 PR (Figures 3a and 3c). In the case of diphtheria toxin we have observed previously that the efficiency of translocation of the A-fragment to the cytosol is higher when the translocation is induced by low pH at the plasma membrane than when translocation occurs from acidic endosomes [26]. We considered it likely that this would also be the case for anthrax toxin, and we therefore performed an experiment that was identical to that described above, except that the incubation of the cells at 37 °C was preceded by a brief incubation with low-pH buffer. The results (Figures 3b and 3d) were similar to those obtained when the low-pH treatment was omitted, except that when FLD/MM and FLD/YV were pretreated with HIV-1 PR, more protein was observed at later time points, probably due to higher translocation efficiency.

Increased ability of toxins to inhibit cellular protein synthesis after HIV-1 PR pretreatment

We have shown previously that decreasing the intracellular stability of DTA also decreases its toxic effect, and it was thus likely that cleavage of FLD/MM and FLD/YV with HIV-1 PR would increase the toxicity of these toxins. We incubated HeLa P4/CCR5 cells overnight with toxins and subsequently measured the ability of the cells to incorporate [^{3}H]leucine. The results (Figure 4) showed that pretreatment with HIV-1 PR substantially increased the toxic effect of FLD/MM and FLD/YV, but not that of FLD, and this increase was abrogated when the HIV-1 PR inhibitor, saquinavir, was present during protease treatment. The stable precursor forms (pre-FLD, pre-FLD/MM and pre-FLD/YV) of the toxins were considerably more toxic than the unstable toxins, and showed toxicities similar to FLD/MM and FLD/YV that had been activated with HIV-1 PR. Thus there seems to have been a good correlation between the stabilities of the toxins and their toxic effects, indicating that the stabilization of a toxin by HIV-1 PR indeed can be used to increase its toxic potential.

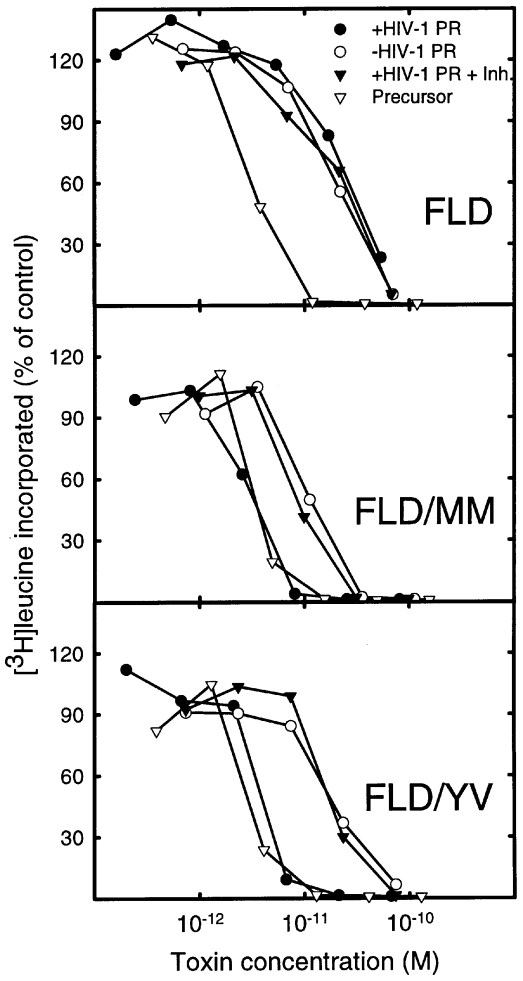

Figure 4 Increased cytotoxicity of sitoxins by treatment with HIV-1 PR

PA (2×10^{-8} M) and increasing concentrations of FLD, FLD/MM or FLD/YV or their precursor forms (pre-FLD, pre-FLD/MM or pre-FLD/YV) were added to the growth medium of HeLa P4/CCR5 cells, which were subsequently incubated overnight at 37 °C. Where indicated, the proteins FLD, FLD/MM and FLD/YV had been pretreated with HIV-1 PR in the presence (+Inh.) or absence of saquinavir. The cells were then allowed to incorporate [^{3}H]leucine for 30 min, and the cell-associated TCA-precipitable radioactivity was measured by scintillation counting.

Inability of sitoxins to specifically inhibit protein synthesis in HIV-infected cells

We wanted to analyse whether sitoxins were able to act preferentially on HIV-1-infected cells by being specifically cleaved and thereby activated by HIV-1 PR. To this end, uninfected and infected HeLa P4/CCR5 cells were treated with the toxins. These cells contain the β-galactosidase gene under the control of the Tat-inducible HIV-1 LTR (long terminal repeat) promotor and infected cells can be detected by blue staining. Cultures of HeLa P4/CCR5 cells, in which 50% of cells were infected by HIV-1 strain NL4-3, were incubated with PA and increasing concentrations of sitoxins (FLD/MM and FLD/YV) overnight. Subsequently, cellular and viral proteins were metabolically labelled with [^{35}S]methionine/cysteine. Under these conditions, specific cleavage and activation of sitoxins by HIV-1 PR should have selectively inhibited protein synthesis in infected cells but not in uninfected cells. Thus overall synthesis of viral proteins would be more strongly inhibited than synthesis of cellular proteins. Total cellular protein synthesis was measured by scintillation counting following TCA precipitation of proteins from cell lysates. To measure HIV-specific protein synthesis, radiolabelled Gag proteins were immunoprecipitated with a specific antiserum against the viral CA protein. Following separation by SDS/PAGE, immunoreactive proteins were quantified by PhosphorImager analysis.

These experiments showed that HIV-specific and total protein synthesis in infected cells were affected to a similar degree by the sitoxins (Figure 5). Preactivation of sitoxins with HIV-1 PR resulted in an increased protein-synthesis inhibition in infected cells that was similar to that observed in uninfected cells (compare Figures 4 and 5). Furthermore, no specific effect on virus-specific protein synthesis was observed when FLD/MM or FLD/YV that had not been preactivated by PR was exposed to infected cells, and HIV-specific and total protein synthesis displayed virtually identical concentration curves with respect to inhibition by the sitoxins (Figure 5). Similarly, no selective eradication of HIV-1-infected cells was observed in sitoxin-treated cultures upon β-galactosidase staining *in situ* and microscopy analysis (results not shown).

The lack of specific toxicity towards HIV-1-infected cells could have been due to the rate of cleavage by HIV-1 PR being slower than the degradation rate of the toxin, resulting in the majority of toxin being degraded. We therefore performed biochemical analysis of sitoxin cleavage by HIV-1 PR in acutely infected HeLa P4/CCR5 cells using either unstable sitoxins (FLD/MM

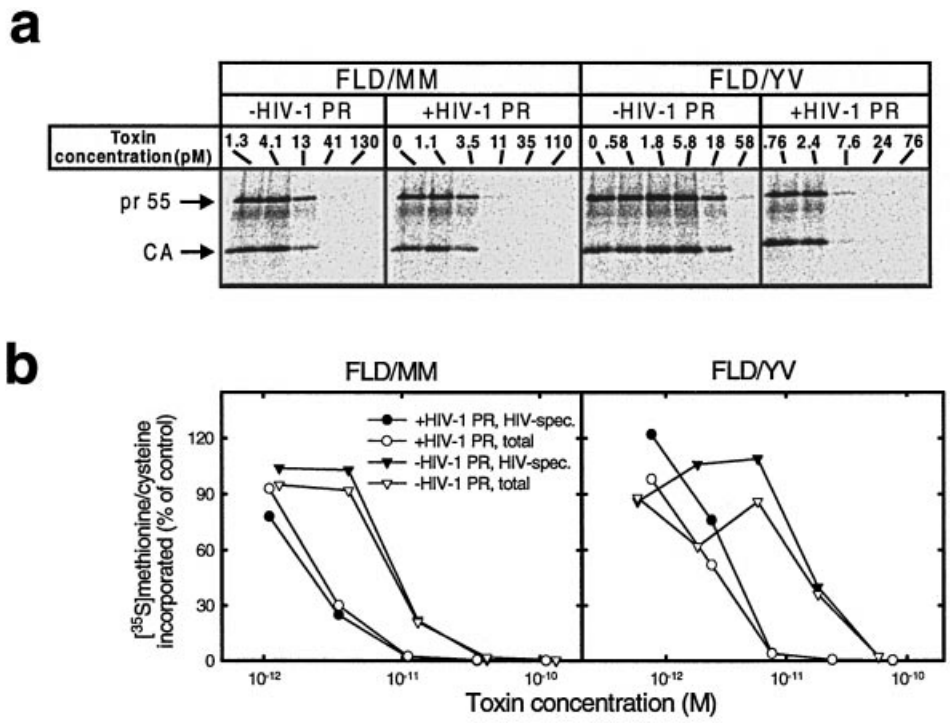

Figure 5 Inhibition of total and HIV-specific protein synthesis in HIV-1-infected cells by sitoxins

HeLa P4/CCR5 cells infected with the HIV-1 strain NL4-3 were incubated for 16 h with PA and increasing concentrations of the toxins, which had been pretreated with HIV-1 PR, where indicated. Subsequently, the cells were labelled with [^{35}S]methionine/cysteine as described in the Experimental section. (**a**) The cell lysate was immunoprecipitated with a polyclonal rabbit antiserum against HIV-1 CA protein. The immunoprecipitates were separated by SDS/PAGE and Phosphor storage screens were exposed to dried gels. (**b**) The intensities of the bands shown in (**a**) representing the CA and the Gag polyprotein (pr 55) were quantified using a Fuji BAS 2000 bioimager, and their sum used as a measure of HIV-specific protein synthesis. Total protein synthesis was measured by scintillation counting following TCA precipitation of an aliquot of the cell lysate that had been removed prior to immunoprecipitation.

and FLD/YV) or their stable precursor forms (pre-FLD/MM and pre-FLD/YV). No processing was observed for up to 7 h after toxin binding (results not shown).

DISCUSSION

Several previous studies have investigated the possibility of constructing toxins that are able to specifically eradicate HIV-infected cells (reviewed in [27]). The dominating strategy has been to target a toxic domain to the surface of the infected cells by fusing it to CD4, which is able to bind to the viral glycoprotein gp120. In the present work we have attempted to construct targeted toxins by using an approach fundamentally different from the one used previously. Cell-specific modulation of the activity of protein toxins by degradation signals that are active only in certain cell types has been suggested previously by Varshavsky [12]. Toxins that are activated through removal of degradation signals by proteases expressed only in certain cells, e.g. viral proteases that are expressed in infected cells, were denoted sitoxins. In the present article we have described the construction of two sitoxins that each contain a different cleavage site for HIV-1 PR.

Initially we were somewhat concerned that LF_N or DTA may themselves contain cleavage sites for HIV-1 PR, since this protease is rather promiscuous and has been reported to cleave a number of non-viral substrates (reviewed in [28]). Also, one of the reported substrates is *Pseudomonas* exotoxin, which works by the same enzymic mechanism as diphtheria toxin. However, we observed that the sitoxins were efficiently cleaved by HIV-1 PR at the introduced sites, and we could not detect any cleavage at other locations at the concentration of HIV-1 PR required to obtain complete cleavage at the new sites. From this we concluded that LF_N and DTA are poor substrates for HIV-1 PR, and that these proteins therefore are well suited for use in sitoxins that contain cleavage sites for this protease.

We observed that treatment with HIV-1 PR converted the sitoxins from unstable proteins with an approximate cellular half-life of 20 min into proteins that were not significantly degraded during the 4-h period of the experiment. This shows that the concept of stabilizing a protein through specific proteolytic removal of a degradation signal works in practice. Also, the results are in good agreement with our previous study on diphtheria-toxin mutants [13], where we found that Phe-FLAG–DTA displayed a half-life of approximately 30 min in Vero cells, whereas the corresponding proteins with stabilizing N-terminal residues had half-lives ranging from 8 to 20 h. All four proteins analysed (FLD, FLD/MM, FLD/YV and Phe-FLAG–DTA), which each contained an N-terminal Phe-FLAG sequence with different following amino acids, were degraded rapidly in cells. These results suggest that the Phe-FLAG sequence may be useful as an efficient degradation signal that can be grafted on to heterologous proteins and used to target them for degradation.

Treatment of the sitoxins with HIV-1 PR resulted in a marked increase of their relative cytotoxic effects, demonstrating that cleavage of an engineered bacterial toxin by HIV-1 PR leads to toxin activation. The processing of the sitoxin FLD/YV by HIV-1 PR resulted in a 10-fold increase in its toxicity, whereas the

corresponding increase in stability seemed to be somewhat higher: the cellular half-life of the protein was altered from 20 min to considerably longer than 4 h. However, a similar result was observed in the case of diphtheria-toxin mutants, where a given change in stability had a smaller effect on cytotoxicity [13]. It has been demonstrated that one molecule of DTA introduced into the cytosol of a cell is sufficient to kill that cell [29], and it may be that a moderate reduction in A-fragment stability will not be sufficient to alter its toxicity.

Although the sitoxins were cleaved by HIV-1 PR *in vitro*, leading to increased stability and cytotoxicity, they did not appear to be processed in HIV-infected cells, and we could not detect any selective eradication of infected cells. One possible reason for this lack of effect could be that binding or intracellular transport of toxin is compromised in the infected cells. However, when we analysed protein-synthesis inhibition by HIV-1 PR-preactivated sitoxins in infected cells, we observed that the synthesis of HIV-specific proteins and overall protein synthesis were equally affected by the sitoxins. This indicates that the ability of the toxin to reach the cytosol was not reduced in the infected cells, and that a reduction in binding or intracellular transport is unlikely to have been the reason why we did not observe any specific effect of sitoxins towards infected cells. Another possible explanation for the lack of specific killing of HIV-infected cells by the sitoxins is that the kinetics of sitoxin cleavage by HIV-1 PR are slow compared with those of sitoxin degradation, leading to stabilization of only a minute fraction of the sitoxin molecules that had been translocated to the cytosol. We could not detect any HIV-1 PR cleavage of the stable sitoxin precursors pre-FLD/MM and pre-FLD/YV in HIV-1-infected cells, indicating that the activity of cytosolic HIV-1 PR was indeed low.

A prerequisite for intracellular cleavage of a substrate is that the enzyme and the substrate are present at the same subcellular location. The sitoxins are likely to be found in the cytosol of the infected cells, since they inhibit protein synthesis in the cytosol, and since they are substrates for N-end-rule-mediated degradation, which is another cytosolic process. HIV-1 PR activity is highest at the site of viral budding and inside the virion, and it is thought that the dimerization of HIV-1 PR that is required for its activity [30] is favoured by the high local concentration of Gag-Pol precursors inside the budding virion. Kaplan and Swanstrom [8] showed that cleavage products of the Gag polyprotein could be recovered from the cytosolic fraction of acutely infected cells, suggesting that some HIV-1 PR activity is present in the cytosol. However, the cytoplasmic activity of HIV-1 PR may be rather low. Whereas many cellular proteins have been reported to be substrates for HIV-1 PR *in vitro*, only a few have been shown to be cleaved in infected cells: the nuclear factor κB precursor [9], actin [10] and vimentin [11]. Two of these are cytoskeletal proteins, which may indicate that HIV-1 PR activity is enriched at the cytoskeleton.

Studies with the HIV-regulatory protein Nef also indicated that the activity of cytosolic HIV-1 PR in infected cells is low. Nef was found to be a good substrate for cleavage by HIV-1 PR *in vitro*. However, analysis of HIV-infected cells showed only uncleaved Nef protein, whereas virion-incorporated Nef was almost completely processed by HIV-1 PR [19,31]. The relative levels of intracellular HIV-1 PR activity may also be affected by the host cell. During preparation of this article, we learned that the HIV-1-infected HeLa P4/CCR5 cells used in this study exhibit a 2–10-fold reduction in intracellular Gag processing compared with established T-cell lines and primary lymphocytes (U. Schubert, personal communication). In summary, low cytosolic HIV-1 PR activity is the most likely reason why no specific elimination of HIV-infected cells by the sitoxins described in this study was observed.

The apoptosis-promoting protein caspase-3 can be cleaved at a specific site by caspase-8, leading to the induction of apoptosis, and during the preparation of this article Vocero-Akbani and co-workers [32] reported the construction of a modified caspase-3 in which this cleavage site had been replaced by a HIV-1 PR site. Also, the 11-amino-acid Tat protein-transduction domain had been added N-terminally in order to transduce this protein into cells, and this modified caspase was able to specifically kill HIV-infected cells. This study showed that the principle of specific eradication of virus-infected cells by an externally added, HIV-1 PR-activatable, 'toxin' works in practice. However, the processing of this protein in infected cells was not analysed biochemically, and it is therefore not known whether the caspase was efficiently cleaved, or if cleavage of only a minute fraction of the total protein by HIV-1 PR was sufficient to provide specific cell killing.

The results described in the present study suggest that it may be difficult to obtain efficient processing of a cytosolic sitoxin in HIV-infected cells. The sitoxins described here displayed a rather moderate toxicity difference (10-fold or less) between the cleaved and the non-cleaved forms, and one would therefore expect a specific eradication of infected cells to depend on a substantial fraction of the toxin being cleaved by HIV-1 PR. However, if one were able to construct a sitoxin where cleavage by HIV-1 PR leads to very large increase in cytotoxicity, such a sitoxin should in theory be able to specifically kill infected cells even if only a minor fraction of total cytosolic sitoxin is actually cleaved by HIV-1 PR.

The enzymic activity of Shiga toxin is increased considerably when the toxin is cleaved at a furin site, leading to the dissociation of the auto-inhibitory A2 moiety from the enzymically active A1 moiety [33]. One may imagine the construction of a toxin that is activated through cleavage by a viral protease at the junction between the enzymic domain and such an auto-inhibitory peptide. Molecular modelling approaches may possibly be used to design peptides that will fit into active sites of toxins, and that can be removed by viral proteases. By using such strategies one may be able to construct toxins where the difference in toxicity between the activated and non-activated forms is so large that efficient eradication of virus-infected cells is accomplished despite low activity of viral protease in the cytosol.

Since HIV buds from the plasma membrane of infected cells, it is likely that the local concentration of HIV-1 PR is high at, or close to, the plasma membrane. The C-terminal 17 amino acids of the K-Ras-4B protein contain two membrane-targeting signals, i.e. a stretch of six consecutive lysines and a farnesylation signal, and this peptide is sufficient to target a heterologous protein to the plasma membrane [34]. Also, we have demonstrated previously that DTA is efficiently farnesylated when a farnesylation signal is attached to its C-terminus [35]. Conceivably, the efficiency of sitoxin cleavage in HIV-infected cells may be increased through the attachment of a membrane-targeting signal, thereby routing the sitoxin to the plasma membrane. One may also introduce a HIV-1 PR cleavage site directly upstream of the C-terminal targeting signal, thereby allowing the toxin to be liberated into the cytosol upon cleavage by the protease.

In summary, our results demonstrate that it is possible to construct a toxin that is specifically activated by a viral protease. The data also suggest that for such a toxin to be able to specifically kill virus-infected cells, the difference in toxicity between the activated and non-activated forms must be larger than for the toxins presented here, or, alternatively, the efficiency of toxin cleavage in infected cells must be increased.

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