HIV Protease-Activated Molecular Switches Based on Beta-Glucuronidase and Alkaline Phosphatase

Taryn L. O'Loughlin and Ichiro Matsumura[*]

Department of Biochemistry, Center for Fundamental and Applied Molecular Evolution, Emory University School of Medicine, Rollins Research Center, Room 4119, 1510 Clifton Road, Atlanta, GA 30322, USA

Abstract: Our long-term goal is to direct the evolution of novel protease variants. To this end we have engineered a new type of protease-activated reporter enzyme. Many protease-activated enzymes evolved in nature, but the introduction of novel regulatory mechanisms into normally unregulated enzymes poses a difficult design challenge. Random Elongation Mutagenesis [1] was used to fuse the p6 peptide, which is recognized and cleaved by HIV protease, and twelve random sequence amino acids to the C-termini of beta-glucuronidase (GUS) and alkaline phosphatase (AP). The resulting GUS-p6-(NNN)$_{12}$ and AP-p6-(NNN)$_{12}$ libraries were expressed in *E. coli* and screened for clones that were inactivated by the C-terminal extension (tail). The inactivated clones were co-expressed with HIV protease, and those that were re-activated were isolated. The AP and GUS activities of the most responsive clones were each >3.5-fold higher when co-expressed with HIV protease, and this activation is correlated with *in vivo* proteolysis. It should be possible to generalize this strategy to different reporter enzymes, different target proteases, and perhaps to other types of protein-modifying enzymes.

Keywords: Molecular switch, biosensor, reporter, random elongation mutagenesis, directed evolution.

INTRODUCTION

The objective of this study is to develop high throughput protease assays for directed evolution studies, but peptide cleavage is difficult to detect. We therefore sought to convert constitutively active reporter enzymes into protease-activated molecular switches. The design of reporters that are inactivated by proteases is relatively straightforward [2-9], but high throughput screens based on inactivation are more likely to lead to the identification of false positives. Previous workers have designed clever molecular switches [10-16], but to our knowledge no one has described a general method to convert unregulated enzymes into zymogens.

Although many protease-activated enzymes exist in nature, the introduction of novel regulatory mechanisms into existing proteins remains a technical challenge to protein engineers. Many eukaryotic proteases are expressed in an inactive form until they are themselves proteolytically processed. The enzyme must initially fold into a particular catalytically inactive conformation and display its pro-peptide correctly. The pro-peptide acts as an auto-inhibitory domain by stabilizing the inactive conformation. Upon proteolytic removal of the pro-peptide, the activity of the protein is restored through a change in conformation. Such systems apparently evolve readily in nature, but are difficult to design by structure-based site-directed mutagenesis.

It seems likely that these molecular switches evolved from ancestors that were not post-translationally regulated. The regulatory mechanism could have evolved in two sequential steps (Fig. **1**). First, recombination events, frame-shift mutations or point mutations in the stop codon extended one copy of the structural gene. A subset of these essentially random sequence extensions reduced the primary catalytic activity of the enzyme, either by occluding the active-site (intra-steric regulation) or stabilization of an inactive conformation (allosteric regulation). Second, the cell evolved novel mechanisms, such as proteolysis, effector-binding and covalent modification, that blocked the intra-molecular interactions of the newly evolved auto-inhibitory domain, such as proteolysis, effector-binding or covalent modification. The capacity to regulate enzyme activities through post-translational modifications would have given the cell a selective advantage over its competitors. The evolution of the auto-inhibited proteins such as importin alpha, kinases, and phosphatases [17] might also have followed this three-step scheme.

Our plan was to create protease-activated reporters by recapitulating this hypothetical evolutionary process *in vitro*. We were inspired by a Random Elongation Mutagenesis (REM) study that showed that proteins are stabilized by selected peptide fusions [1]. We reasoned that some C-terminal peptide fusions could decrease activity, either by intrasteric or allosteric regulation, and that these novel auto-inhibitory activities could in turn be alleviated by proteolytic removal. Here we describe the REM of two dissimilar reporter genes and the identification of protease-activated variants.

MATERIALS AND METHODS

Materials

Escherichia coli strain InvaF' was purchased from Invitrogen (Carlsbad, CA). *E. coli* E15 (*phoA8(del), garB10, fhuA22, ompF627(T2R), fadL701(T2R), relA1, pit-10, spoT1, rrnB-2, mcrB1, creC510*) was obtained from the *E. coli* Genetic Stock Center, and converted into E15 (DE3) with

*Address correspondence to this author at the Department of Biochemistry, Center for Fundamental and Applied Molecular Evolution, Emory University School of Medicine, Rollins Research Center, Room 4119, 1510 Clifton Road, Atlanta, GA 30322, USA; Tel: (404) 727-5625; Fax: (404) 727-3452; E-mail: imatsum@emory.edu
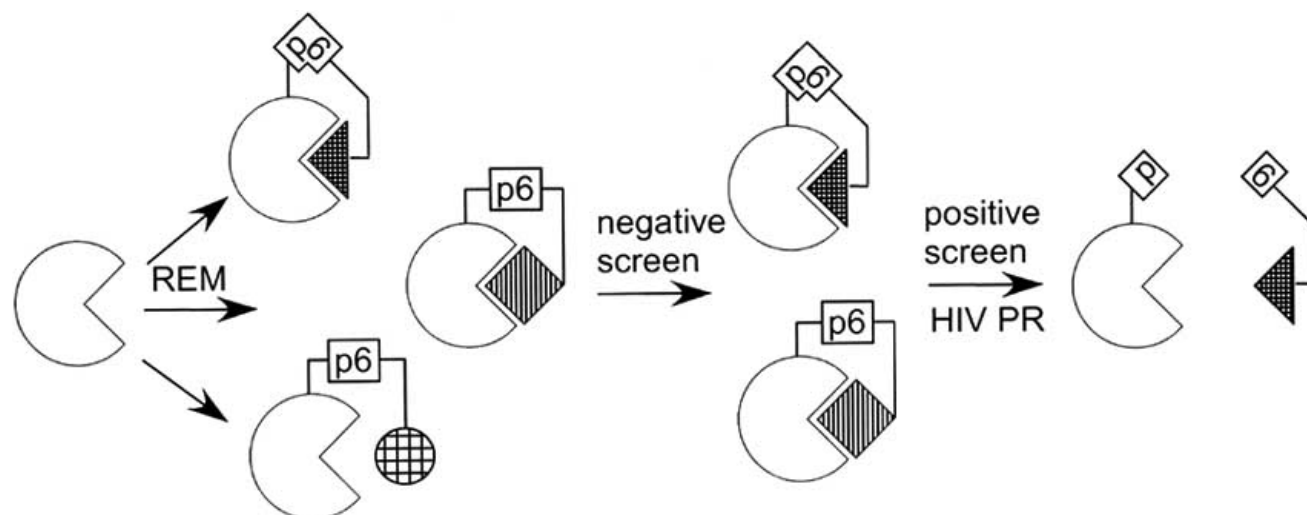
Fig. (1). Evolution of novel auto-inhibitory domains. Ancient enzymes (represented as empty semi-circles with triangular active-sites) were probably constitutively active. Mutations and recombination events (Random Elongation Mutagenesis) fused random sequence peptides to the termini (represented as filled geometric shapes). Post-translational regulation evolved through selection against constitutively active variants (circle). Constitutively inactive variants (square, as HIV protease recognizes p6 peptide sequence only in bent conformation) were also disadvantageous.

the Lambda DE3 Lysogenation Kit (Novagen, Madison, WI). The *lacI* expression vector pREP4 was from Qiagen (Chatsworth, CA), pBAD myc his A was from Invitrogen, and pSL1180 was from Amersham/GE Healthcare. pCDF-Duet and pET28a(+) were from Novagen (Madison, WI). DNA sequencing kits were from Perkin-Elmer/Applied Biosystems (Foster City, CA). DNA purification columns were purchased from Qiagen (Chatsworth, CA). The Butterfly nitrocellulose membranes were from Schleicher and Schuell (Keene, NH). The histochemical GUS substrate, 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide (X-gluc), and the inducer isopropyl-beta-D-thiogalactopyranoside (IPTG) were from Gold Biotechnology (St. Louis, MO); the histochemical AP substrate, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), was from Sigma Chemicals (St. Louis, MO). All DNA modifying enzymes (restriction enzymes, taq polymerase, T4 DNA ligase) were from New England Biolabs (Beverly, MA). Oligonucleotides were synthesized by IDT (Coralville, IA). Primary antibodies were from Abcam (Cambridge, MA) and secondary antibodies were from Rockland Immunochemicals (Gilbertsville, PA).

Plasmids and Cloning

The constitutive *E. coli* GUS expression vector, P_{lac*}-6his-*gusA*-pET20, was previously described [18]. We subcloned the p6 (VSFNFPQITL), HA (YPYDVPDYA) and $(NNN)_{12}$ in two consecutive whole plasmid PCR reactions [19]. First, we used primers HA-pET30 and N36-3'pETout2 (Table **1**) to amplify P_{lac*}-6his-*gusA*-pET20. The PCR product was purified, self-ligated and electroporated into InvαF'/pREP4. We purified the resulting P_{lac*}-6his-*gusA*-HA-$(NNN)_{12}$-pET20 plasmid library and PCR amplified it using primers p6-3'pET and SalI-HA. Again the PCR product was purified, self-ligated and electroporated into InvαF'/pREP4. The transformants were propagated in LB-ampicillin/kanamycin, and the P_{lac*}-6his-*gusA*-p6-HA-$(NNN)_{12}$-pET20 plasmid library was purified.

The inducible HIV protease expression vectors, p1+IQ (*lacZ*) and P_{BAD}-HIV PR-pACYC184 were constructed as follows. The expression vector p1+IQ[5] was obtained from the American Type Culture Collection. The *lacZ* gene was excised from this vector using Bam HI; the remaining DNA was purified, self-ligated and used to transform *E. coli* strain InvαF'. The HIV protease gene was subcloned from p1+IQ (*lacZ*) into pBAD myc his A (Invitrogen) using restriction enzymes Eco RI and Hind III. The *araC*-P_{BAD}-HIV PR expression cassette was subcloned from HIV PR-pBAD plasmid into pACYC184 (New England Biolabs) using Sph I and Sal I.

The P_{BAD}-HIV PR-pCDF expression vectors were constructed in two stages. First, we made the P_{BAD}-pCDF expression vector by subcloning the *araC* repressor and P_{BAD} promoter from pBAD myc his A into pSL1180 using Sph I and Nco I; the P_{BAD} promoter was then subcloned from P_{BAD}-pSL1180 into pCDF Duet using Nco I and Xba I. Second, the HIV protease gene in p1+IQ was PCR amplified using primers 5'NdeI-HIVpr and pUC-600, subcloned into pET28a+ using Nde I and Hind III and sequenced to confirm its wild-type identity. The subcloning fused DNA encoding a hexa-histidine tag to its 5' end. The inactivating D25N mutation was introduced into 6his-HIV PR-pET28 by whole circle PCR using primers HIVPR-D25N-62 and HIVPR-62out. The mutant gene was also sequenced to confirm its identity. The wild-type and D25N variants of the 6his-HIV PR gene were subcloned from their respective 6his-HIV PR-pET28 plasmids into P_{BAD}-pCDF using Nco I and Xho I.

The *phoA* gene was amplified from the chromosome of *E. coli* using the primers, 5'-phoA and 3'phoA (Table **1**), and cloned into pET28 using NdeI and HindIII. The 3'spacer-p6-*phoA* and N36-3'-pET-out primers were used to amplify the entire *phoA*-pET28a+ plasmid. The PCR product was purified, self-ligated and electroporated into *E. coli* InvαF'. *E. coli* strain E15(DE3) was transformed with the

Table 1. **Sequences of Primers Used in this Study**

Primer Name	Sequence (5' to 3')	Purpose
HA-pET30	ccccgctagcataatccggcacatcatacgg atacgcaagcttgtcgacggagctcgaa	Constructing P_{lac*}-6his-*gusA*-p6-HA-(NNN)$_{12}$-pET20
N36-3'pETout2	ccccgctagc (nnn)12 tagcggccgca ccaccaccactgagatccggct	Constructing P_{lac*}-6his-*gusA*-p6-HA-(NNN)$_{12}$-pET20
p6-3'pET	ggcgtcgactatccgtatgatgtgccggattatgct	P_{lac*}-6his-*gusA*-p6-HA-(NNN)$_{12}$-pET20 library
SalI-HA	ggcgtcgactatccgtatgatgtgccggattatgct	P_{lac*}-6his-*gusA*-p6-HA-(NNN)$_{12}$-pET20 library
5'NdeI-HIVpr	ctaccattaccagttggtctggtgtca	Cloning of HIV PR
pUC-600	gaaaacgttcttcggggcgaa	Cloning of HIV PR
HIVPR-D25N-62	gaagctctattaaatacaggagcagatg	Cloning of D25N HIV PR
HIVPR-62out	cttttagttgccccccctatctttattgtg	Cloning of D25N HIV PR
5'phoA	gggtctagacatatgaaacaaagcactattgcactggcac	Amplification of phoA from *E. coli* chromosome
3'phoA	cccaagcttgagatctttcagccccagagcggctttcatgg	Amplification of phoA from *E. coli* chromosome
3'p6-spacer-phoA	cgctagcgtgatctgcgggaagttgaagctcacggtcgcat catccgggaagctctcgagtgcggccgcaagcttgaga	*phoA*-p6-(NNN)$_{12}$-pET28 library construction
N36-3'pETout	(nnn)12 tgagatccggctgctaacaaagcccga	*phoA*-p6-(NNN)$_{12}$-pET28 library construction
3'pET3	gcatgccgtaaagcactaaatcggaacc	Sequencing of gusA and phoA tails
gusA-1440	tatgtccaaagcggcgatttggaaacg	Sequencing of gusA tail
phoA 1325	acataccggcagtcagttgcgtattgc	Sequencing of phoA tail

resulting *phoA*-p6-(NNN)$_{12}$-pET28 plasmid library was purified and transformed into E15(DE3) for the high throughput screen.

DNA Sequencing

The most responsive clones, *gusA* (clone 461) and *phoA* (clone 1), were sequenced using the primers 3'pET for both 3'ends and gusA1440 and phoA-1325 for the 5' ends, respectively. We employed the ABI BigDye 3.1 protocol, and sequencing reaction products were separated and analyzed at the Emory/Georgia Tech FAME Center. To obtain the sequence of phoA (clone 1), we tried numerous sequencing reaction conditions and outsourced the job to SEQWRIGHT (Houston, TX), a company that specializes in the sequencing of difficult templates, including those that are GC-rich.

Western Blotting

Cultures containing *gusA* (clone 461) and *phoA* (clone 1) were expressed as described for the enzyme assays. One milliliter of culture was centrifuged for 5 minutes on a tabletop microfuge to collect the cells. The supernatant was discarded and the cells were resuspended in 100 microliters of 1x sodium dodecyl sulphate (SDS) buffer. Samples were then boiled and spun again. The protein within each 10 microliter sample was separated in a 6% polyacrylamide protein gel and transferred onto a nitrocellulose membrane. After transfer, the membrane was blocked for one hour using 4% non-fat milk dissolved in phosphate buffered saline (PBS). The membrane was then probed with one of three primary antibodies for one hour. A polyclonal rabbit anti-GUS antibody diluted 1:7,000 and a monoclonal mouse anti-HA antibody diluted 1:10,000 was used to detect the engineered GUS, and a monoclonal mouse anti-AP antibody diluted 1:10,000 was used for the engineered AP. The membrane was then washed 4X with PBS containing 0.1% Tween-20 and probed with goat anti-rabbit or goat anti-mouse IRD labeled antibodies diluted 1:10,000. After washing again 4X with PBS containing 0.1% Tween-20, the immobilized antibody-antigen complexes were visualized and quantified on a LI-COR Odyssey Infrared imager.

RESULTS

The technical endpoint of this study is to attach cleavable auto-inhibitory domains to two *E. coli*-derived reporter enzymes, GUS and AP. These reporters are good starting points because they are commonly used, well characterized and different enough from each other to demonstrate that our approach is not restricted to any particular protein fold. It would be difficult to predict the exact amino acid sequence of a cleavable auto-inhibitory peptide, so random mutagenesis and screening were employed. Different vectors and *E. coli* strains were used for the over-expression of *gusA* and *phoA*, respectively, but the overall strategy was identical. Random sequences were inserted at the C-termini of both genes by an inverse PCR method called "whole plasmid PCR" [19], and screened for clones that were active

only when co-expressed with Human Immunodeficiency Virus protease (HIV PR). This protease is a good model because it is well characterized, can be expressed in *E. coli* [5], and is relevant to human disease.

A constitutive GUS expression vector, P_{lac*}-6his-*gusA*-pET20 [18] was used as an ancestral (starting) plasmid. DNA encoding three consecutive peptides was inserted just upstream of the 3' stop codon in sequential whole plasmid PCRs [19] (Fig. **4a**). The first peptide was p6 (VSFNFPQITL), which when displayed in the correct orientation is efficiently cleaved by HIV PR [5]. The second

was the HA epitope (YPYDVPDYA), which could be used to monitor protein expression and cleavage. The third was degenerate $(NNN)_{12}$ sequence encoding twelve random amino acids.

E. coli InvαF' cells were co-transformed with the P_{lac*}-6his-*gusA*-p6-HA-$(NNN)_{12}$-pET20 library and the inducible HIV protease expression vector, P_{BAD}-HIV PR-pACYC184. The transformed cells were propagated on LB agar plates supplemented with 50 micrograms/mL ampicillin and 34 micrograms/mL chloramphenicol and 80 micrograms/mL X-gluc. The GUS variants were expressed under these
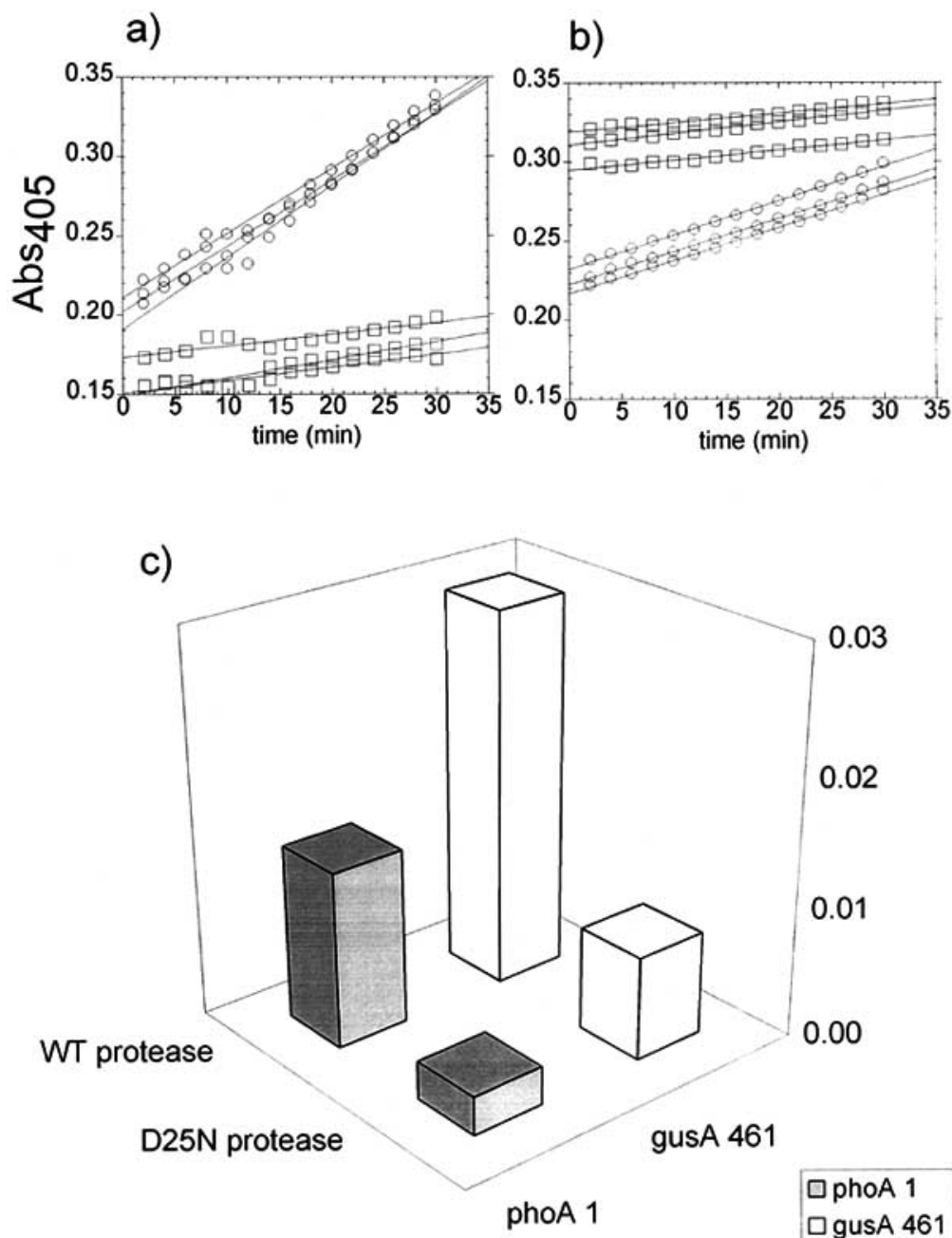

Fig. (2). HIV protease activates beta-glucuronidase and alkaline phosphatase variants. E. coli cells were propagated and induced to co-express a reporter (selected beta-glucuronidase (A) or alkaline phosphatase (B)) variant AND HIV protease (wild-type = ○; catalytically inactive D25N = □). The intact cells were reacted with pNP-glucuronide (A) or pNP-phosphate (B), which were apparently cell-permeable; the formation of the pNP product was followed in a microplate spectrophotometer at 405 nm. The rates of production formation were calculated from the slopes of the lines in plots 2A and B (C).

conditions; HIV PR was not. We examined ~20,000 colonies and picked ~400 that exhibited diminished GUS activity (lighter blue) relative to the parental control colonies. The latter were re-streaked on LB-amp X-gluc plates and identical plates supplemented with 0.2% L-arabinose, which induced the expression of HIV protease. Thirteen clones exhibited greater GUS activity on the plates supplemented with 0.2% L-arabinose than on the plates lacking this inducer, and one of these (*gusA*-p6-HA-(NNN)$_{12}$ clone 461) reproducibly exhibited the most pronounced HIV protease-dependent activation.

To quantify the HIV protease-dependence, InvαF' cells were co-transformed with the selected (clone 461) P$_{lac*}$-6his-*gusA*-p6-HA-(NNN)$_{12}$-pET20, and either P$_{BAD}$-WT HIV PR-pCDF or P$_{BAD}$-D25N HIV PR-pCDF. The latter vectors produce the wild-type or a catalytically inactive (but correctly folded) variant of HIV protease [21], but are otherwise identical. The transformed cells were propagated to mid-log phase in liquid LB medium supplemented with 50 micrograms/mL ampicillin and 50 micrograms/mL spectinomycin. Gene expression was induced for three hours with 0.5 mM IPTG and 0.2% L-arabinose. We added 50 microliters of each culture (in triplicate) to 250 microliters 2 mM para-nitrophenyl-beta-D-glucuronide in 50 mM Tris, pH7.6. The chromogenic substrate is able to permeate the intact cells and react with the GUS [20]. The formation of the para-nitrophenol product was followed in a microplate spectrophotometer (405 nm) for 30 minutes (Fig. **2a**). The cells expressing the *gusA*-p6-HA-(NNN)$_{12}$ and wild-type HIV protease exhibited >3.5-fold more GUS activity than isogenic cells expressing the D25N protease mutant (Fig. **2c**). These results show that the GUS activity of *gusA*-p6-HA-(NNN)$_{12}$ clone 461 is dependent upon the catalytic activity of HIV protease rather than some other indirect mechanism.

Western blot analysis showed a correlation between cleavage of the p6 site *in vivo* and increased GUS activity (Fig. **3a**). Antibodies specific to GUS and to the C-terminal HA tag, each associated with secondary antibodies conjugated to unique dyes (one that emits infrared light at 700 nm and one that emits at 800 nm), were used to observe cleavage of the p6 peptide in the same gel. The first panel in Fig. **3a** shows a blot with the polyclonal anti-GUS antibodies. The band corresponding to GUS (~72kDa) is shown; other non-specifically reacting bands were eliminated for clarity. A doublet can be seen in the lane where gusA 461 is coexpressed with WT HIV PR, indicating cleavage of the roughly 3kDa "tail". The second panel shows a blot with the anti-HA antibody and, though equal volumes of cells were loaded, there is clearly a decrease in the intensity of this band when coexpressed with WT HIV PR. Since the p6 region is present upstream of the HA sequence in the tail region, cleavage of p6 would eliminate the HA epitope from the tail. Finally, the last panel of this figure is a merge of the two antibodies. The doublet can again be seen in the lane with WT HIV PR, only now it is clear that the top band corresponds to the unaltered engineered protein, containing the HA tag and the bottom band represent the cleaved GUS. This result, combined with the activity assays, shows that cleavage at the p6 site correlates with increased GUS activity.

An alkaline phosphatase (AP) based HIV protease sensor was also created to demonstrate that the approach can be generalized to other reporter enzymes. Whole plasmid PCR was used to create a *phoA*-p6-(NNN)$_{12}$-pET28 library. The library was electroporated into *E. coli* E15 (DE3) cells, which lack their chromosomal *phoA* genes. The transformed cells were propagated on LB-kanamycin agar plates. The resulting colonies were adsorbed to nitrocellulose membranes and transferred colony-side up to LB-kanamycin plates supplemented with 0.5 mM IPTG and 80 micrograms/mL BCIP. We screened ~3000 colonies and identified six hypomorphs (less AP activity than wild-type control colonies). These were re-streaked onto LB-kanamycin plates, and filter-lifted onto LB-kanamycin, 0.5 mM IPTG, 80 micrograms/mL BCIP agar plates. The colonies derived from one of the six isolates (clone 1) consistently turned blue very slowly (~12 hours at 37 degrees, compared to 30 minutes for the wild-type *phoA* control), and this single clone was selected for further characterization.

E. coli E15 (DE3) cells were co-transformed with the selected (clone 1) *phoA*-p6-(NNN)$_{12}$-pET28 plasmid and either P$_{BAD}$-WT HIV PR-pCDF or P$_{BAD}$-D25N HIV PR-pCDF. The transformed cells were propagated in LB-kanamycin/spectinomycin media to mid-log phase, and the proteins were induced with 0.5 mM IPTG and 0.2% L-arabinose for three hours. The cells were reacted with para-nitrophenyl-phosphate, and the formation of the pNP product was followed in a spectrophotometer for 30 minutes (Fig. **2b**). The cells expressing the AP-p6-(NNN)$_{12}$ and wild-type HIV protease exhibited almost 5-fold more activity than isogenic cells expressing the catalytically inactive D25N protease (Fig. **2c**). These results demonstrate that the AP activity of clone 1 (like that of GUS clone 461) is dependent upon protease activity.

Western blot analysis was again used to monitor proteolysis of the engineered AP *in vivo*. *E. coli* cultures expressing AP clone 1 and either the WT or D25N HIV protease were lysed, and the protein was separated by SDS-PAGE and probed with a monoclonal anti-AP antibody (Fig. **3b**). The engineered protein should run at about 50.5kDa and cleavage at the p6 site cuts off about 2kDa, leaving a product of 48.5kDa. A cleavage product can be seen in the lanes expressing the engineered protein along with the WT HIV PR of the expected size. The lower molecular weight of AP makes the cleavage product even easier to visualize than that of the GUS variant. These results therefore indicate a correlation between HIV PR activity, cleavage of the AP protein at the expected site and increased enzyme activity.

The *gusA*-p6-HA-(NNN)$_{12}$ (clone 461) and *phoA*-p6-(NNN)$_{12}$ (clone 1) genes were sequenced. The *gusA* variant included the expected p6 and HA sequences, followed by PIVLCVGVYIYL (Fig. **4a**). These peptides (including spacers) add a total of 38 hydrophobic amino acids to the C-terminus of GUS. We attempted to sequence the *phoA*-p6-(NNN)$_{12}$ gene, but found that the otherwise high quality sequence stopped precisely where the (NNN)$_{12}$ sequence was expected to start (Fig. **4b**). This result is reproducible for both strands of DNA, even when it is encoded in a small
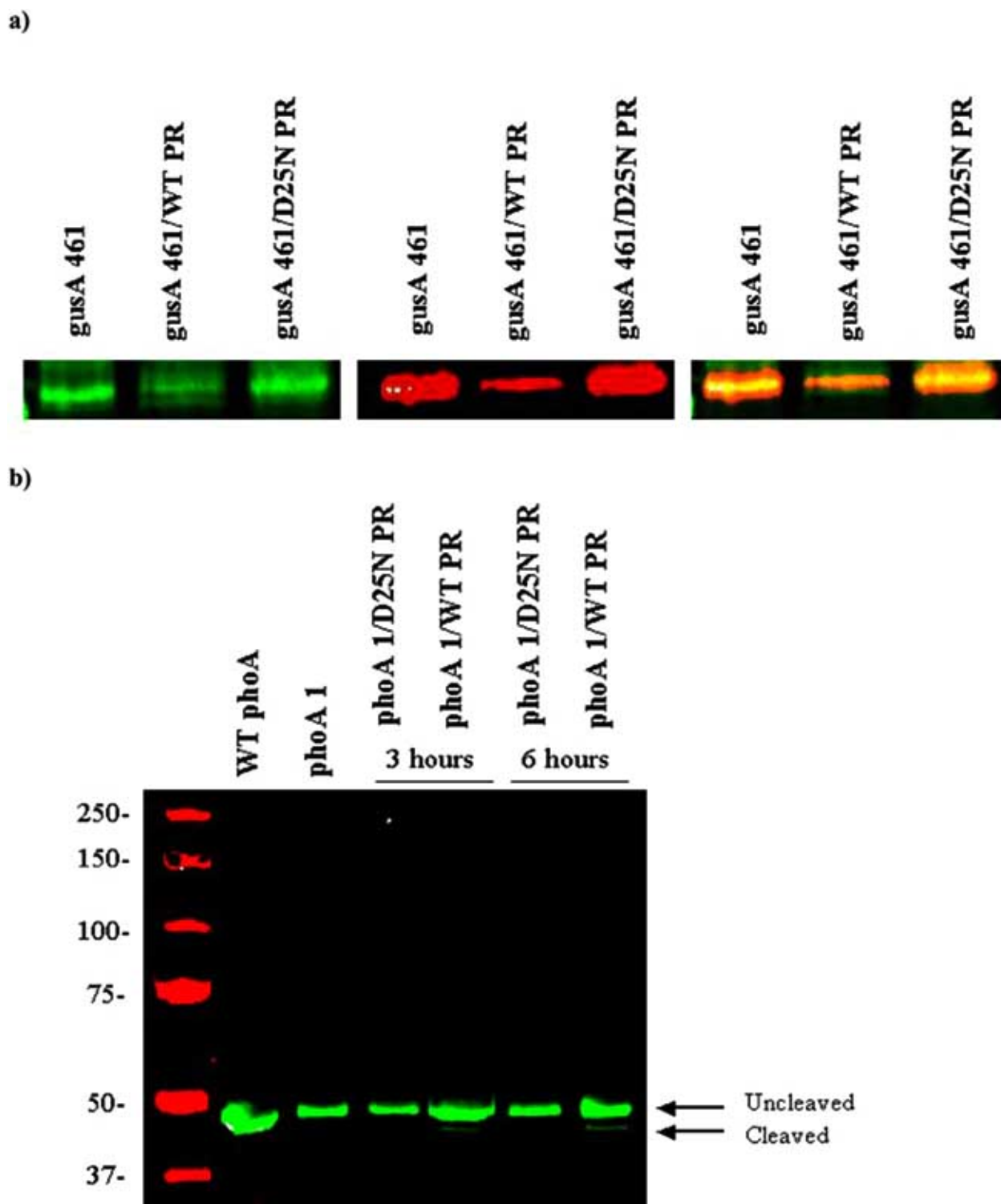
a)

b)

Fig. (3). Western blots showing cleavage of the engineered reporter proteins. (A) A western blot was performed with anti-GUS (green) antibodies and anti-HA (red) antibodies. The left panel is a blot probed for GUS alone, the middle is probed for HA alone and the right-most panel shows a merge of the two images. The arrow and white box represents the expected size of GUS. (B) A western blot was performed with anti-AP antibodies. The blot shows a clear cleavage product when the engineered AP is coexpressed with WT HIV PR.

PCR fragment. These results suggest to us that the $(NNN)_{12}$ sequence has significant secondary structure at the 65 degree temperature of the sequencing reaction.

DISCUSSION

The whole cell assays and Western blot experiments establish a correlation between HIV protease expression, proteolysis at inserted p6 sites and increased reporter enzyme
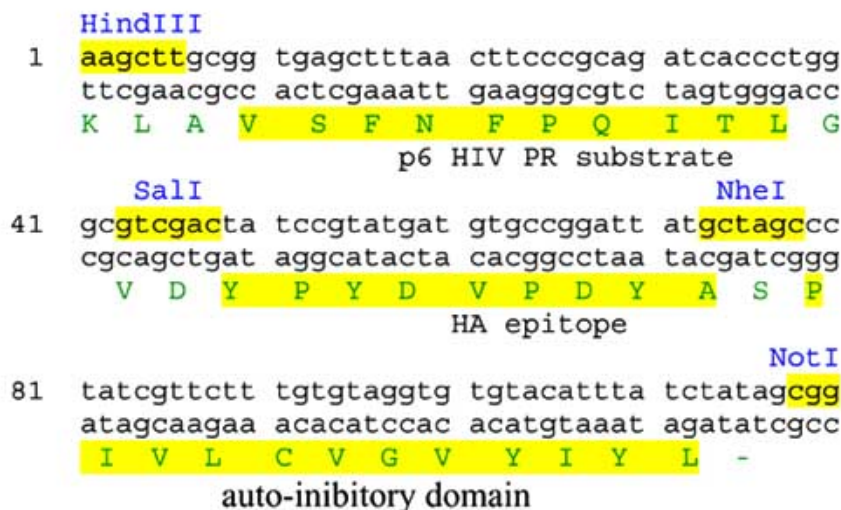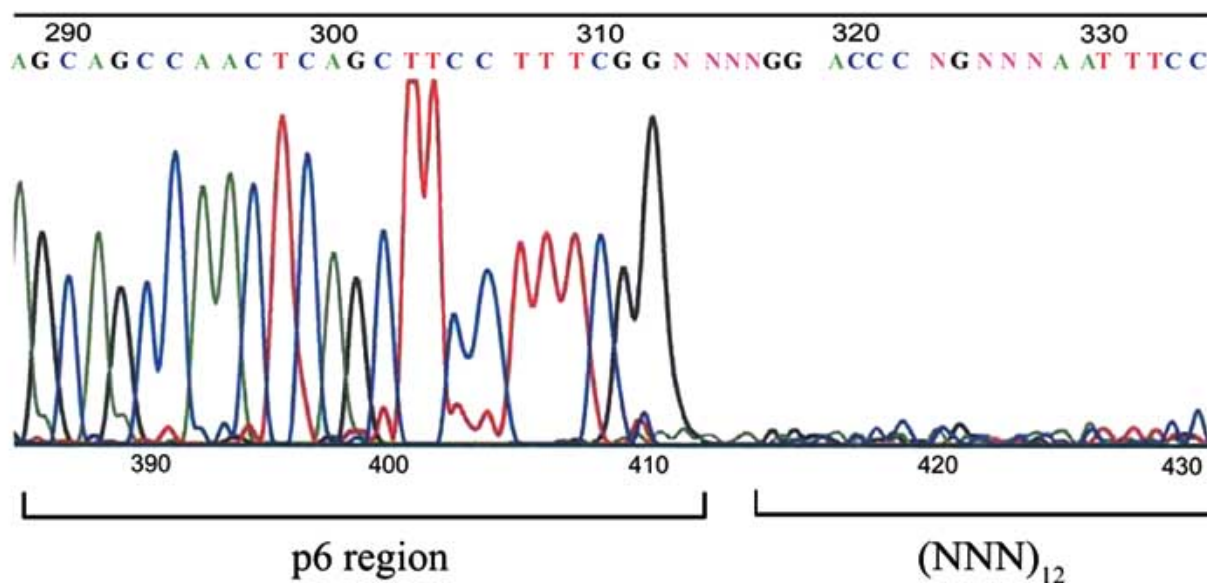
Fig. (4). Sequencing of the engineered reporter proteins (A) p6-HA-(NNN)$_{12}$ fused to beta-glucuronidase (B) A chromatogram showing high quality sequencing halting just upstream of the random nucleotides fused to AP.

activity. It is not clear, however, how the selected C-terminal peptides inhibit the activity of the reporter enzyme prior to proteolysis. The peptide extensions might occlude the active-site, as shown in Fig. **1**. Alternatively, they might destabilize the entire fusion protein, or in the case of alkaline phosphatase interfere with proper secretion. We have purified HIV protease, and are working to purify the GUS-p6-HA-(NNN)$_{12}$ (clone 461) and AP-p6-(NNN)$_{12}$ proteins in order to investigate these mechanisms *in vitro*.

We have shown that Random Elongation Mutagenesis and high throughput screening can be used to produce protease-dependent enzyme activities. This approach could likely be extended to other reporters, other proteases and possibly other protein-modifying activities. Ultimately, it could lead to the fabrication of *E. coli* strains that are dependent upon non-native protease activities for survival. Such strains would enable ultra-high throughput genetic selections for the directed evolution of interesting protease variants, or for the identification of novel protease inhibitors.

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