Genetic selection for dissociative inhibitors of designated protein–protein interactions

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Many biological processes rely on protein–protein interactions. These processes include signal transduction, cell cycle regulation, gene regulation, and viral assembly and replication. Moreover, many proteins and enzymes manifest their function as oligomers. We describe here an efficient means to sift through large combinatorial libraries and identify molecules that block the interaction of target proteins in vivo. The power of this approach is demonstrated by the identification of nine-residue peptides from a combinatorial library that inhibit the intracellular dimerization of HIV-1 protease. Fewer than 1 in 10^6 peptides do so. In vitro biochemical analyses of one such peptide demonstrate that it acts by dissociating HIV-1 protease into monomers, which are inactive catalysts. Inhibition is enhanced further by dimerizing the peptide. This approach enables the facile identification of new molecules that control cellular processes.

Keywords: AIDS, chemical genetics, drug discovery, lambda repressor, peptide library

Interactions between proteins play a critical role in cell function and dysfunction. Such interactions can be identified by phage display[1,2] and the yeast two-hybrid system[3–6], as well as by biochemical methods. The identification of a protein–protein interaction is, however, only the first step. Modulation of the interaction is necessary to produce true insight into its biological purpose. Blocking particular protein–protein interactions with specific ligands would enable exquisite control of cellular processes and provide potential leads for novel chemotherapeutic agents[7].

HIV-1 protease is critical to viral maturation and infectivity[8,9], and is a primary target in AIDS chemotherapy[10–12]. To treat AIDS, attempts have been made to inactivate the protease using active site-directed inhibitors. This strategy has led to the appearance of numerous HIV strains with drug-resistant proteases[10,13]. The prevalence of these resistant strains, along with the toxicity of existing drugs, underscores the need for an alternative approach.

Disrupting the quaternary structure of HIV-1 protease is an orthogonal means of inactivating the enzyme[14–25]. HIV-1 protease is composed of two identical 99-residue subunits[26]. Dissociation of the active, dimeric form of the enzyme results in the complete loss of catalytic activity. We sought to identify new molecules that interfere with the dimerization of HIV-1 protease. In doing so, we were able to develop a genetic selection for dissociative inhibitors of virtually any designated protein–protein interaction.

Results and discussion

Strategy. Our approach takes advantage of the properties of the repressor protein from bacteriophage λ (ref. 27). The lambda repressor protein (cI) binds to its operator DNA as a homodimer. Each monomer has two distinct domains[28], an N-terminal DNA-binding domain and a C-terminal dimerization domain. The role of the C-terminal domain of cI can be fulfilled by another protein[29–35] or a peptide[36,37].

We fused a variant protease (D25N) to the N-terminal DNA-binding domain of cI (NcI; residues 1–131) to create a hybrid repressor (NcI-PR). A dimer of the D25N variant is indistinguishable from the native protease, except for its lack of catalytic activity[38]. Because NcI alone is not capable of binding DNA in vivo, the function of NcI-PR relies on dimerization being mediated by its protease domain. We constructed the selection module λP_R-*lacZ*-*tet* in a reporter plasmid that also directs the production of NcI-PR (Fig. 1). We found that NcI-PR and a related hybrid protein (NcI-dPR2) do efficiently repress the transcription of the *lacZ* and *tet* genes, which are under control of the λP_R promoter (Table 1).

We constructed a library of 5×10^8 peptides with nine random residues fused to the solvent-exposed C terminus of *Escherichia coli* thioredoxin (Trx). Trx and the nonapeptide library were separated by a SG_3 tetrapeptide spacer. The genetic encoding of this library allows for its facile creation, maintenance, and reproduction[39]. We chose not to constrain the peptides[5,6,40] so as to avoid an a priori conformational bias that could limit the number of effective inhibitors. When a cell is transformed with the reporter plasmid, NcI-PR represses the transcription of the reporter genes and the transformant shows a $LacZ^-Tet^S$ phenotype. Upon cotransformation with the peptide library plasmid (Fig. 1), cells that bear dissociative peptides should show a $LacZ^+Tet^R$ phenotype, propagating on solid medium containing tetracycline and forming blue colonies. The stringency of selection can be tuned by changing the tetracycline concentration (between 10 and 40 μg ml^{-1}). To screen the library, we transformed bacteria sequentially with the library plasmid and the reporter plasmid, and then selected for tet^R cells (at 20 μg ml^{-1} tetracycline).

Approximately 300 of the 3×10^8 cotransformants showed a $LacZ^+Tet^R$ phenotype, a selection of 1 in 10^6. This low frequency is surprising. Apparently, disrupting the interaction between two proteins, even small ones such as HIV-1 protease monomers, is difficult. The formidable challenge of identifying inhibitors of a protein–protein interaction highlights the need for a facile assay that can sift rapidly through large (>10^8-member) libraries for active molecules. Our approach meets these criteria. In contrast, genetic selections based on eukaryotic cells (such as the yeast two-hybrid system) are impaired by low transformation efficiencies and cannot reliably identify rare (1 in 10^6) events.

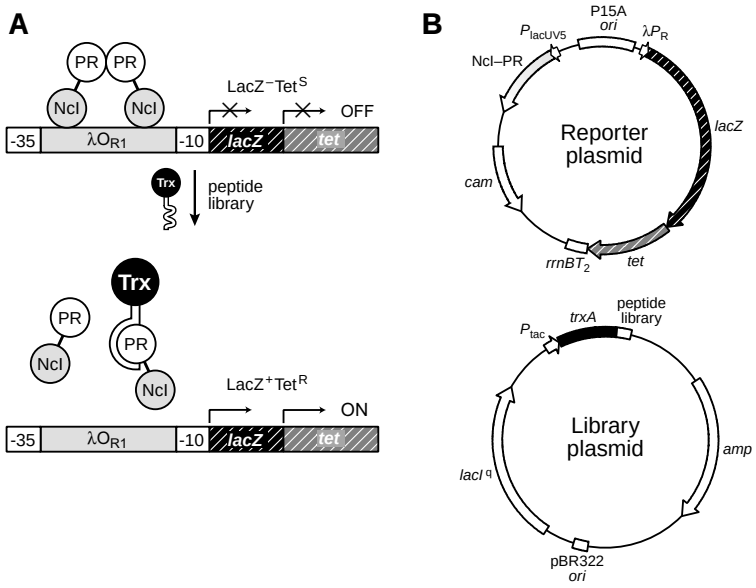

Figure 1. Genetic selection for dissociative inhibitors of a protein–protein interaction. (A) Strategy for selecting peptide inhibitors of HIV-1 protease (PR) dimerization. The transcription of reporter genes is turned on when a peptide fused to thioredoxin (Trx) dissociates the hybrid lambda repressor (NcI-PR). Only LacZ$^+$Tet^R cells survive and form blue colonies in the presence of tetracycline and X-Gal. (B) Map of the reporter plasmid (pSH26) and library plasmid used to select for peptide inhibitors of HIV-1 protease dimerization.

Elimination of false positives. False positive results are common to genetic selections and screens that rely on the transcriptional control of reporter genes. A likely false positive result in our system is a Trx–peptide fusion that interferes with the interaction between λO_{R1} and NcI, and thus results in a LacZ$^+$Tet^R phenotype regardless of the integrity of the protease dimer. We used a gel retardation assay to screen 120 positive isolates from the genetic selection for the ability to interfere with the λO_{R1}–NcI interaction by using a gel retardation assay. Specifically, we mixed lysates from LacZ$^+$Tet^R cells with wild-type cI protein and fluorescein-labeled λO_{R1} DNA, and subjected the mixture to electrophoresis in a polyacrylamide gel. False positives (one-fifth of the cotransformants with LacZ$^+$Tet^R phenotype) cause the shifted band to disappear (Fig. 2A) by interfering with the protein–DNA interaction. Additional gel retardation assays showed that the false positives bind to NcI rather than λO_{R1} (data not shown).

High-throughput ELISA. A molecule that disrupts a dimer would likely have affinity for the interfacial surface of its constituent monomers. We developed a high-throughput screening method to test the ability of Trx–peptide fusions to bind to monomeric protease. Specifically, we covalently immobilized either urea-denatured or native protease on the surface of a maleimide-activated 96-well plate through its two reactive cysteine residues (Cys 67 and Cys 95). Free monomers were removed from immobilized monomers by extensive washing following the alkylation reaction. Positive isolates from the gel retardation assay were tested for the ability to bind to the protease monomer. Bound Trx–peptide fusions were identified by an enzyme-linked immunosorbent assay (ELISA) using fluorescein diphosphate (FDP) as a substrate for the alkaline phosphatase conjugate (Fig. 2B). The affinity of a peptide for the protease should be related to the intensity of the ELISA signal. We identified the nine isolates that produced the strongest signal, and determined the sequence of their encoded peptides. Using immobilized urea-denatured or native protease gave similar results (data not shown).

Analysis of true positives. The sequences of the most effective peptides fall into three groups (Fig. 2C). A common feature in all three groups is the abundance of nonpolar residues, especially valine, alanine, and glycine. This overall composition is similar to that of the dimer interface of HIV-1 protease, which consists largely of an anti-parallel β-sheet composed of the four N- and C-terminal segments of the dimer: P^{1}Q^{2}I^{3}T^4 and T^{96}L^{97}N^{98}F^{99}, respectively[26]. The lack of strong similarity among the nine sequences is not surprising, because the clones are from a single round of selection in vivo rather than multiple rounds of selection in vitro (in which positive clones are enriched in each round[1,2]). Known peptide inhibitors of HIV-1 protease dimerization are based verbatim on the dimeric interface[14–18,20,21,23–25]. In contrast, the sequences from positive isolates (Fig. 2C) do not imitate the protease, and hence are new leads for chemotherapeutic agents.

Dissociative inhibitors of multisubunit enzymes differ in their mechanism of action from that of traditional enzyme inhibitors[7]. An in vitro analysis of inhibition enabled us to distinguish between competitive, uncompetitive, and dissociative inhibition of HIV-1 protease. We synthesized a peptide (pep52) corresponding to a Trx–peptide fusion that gave a strong signal in the ELISA. We found that pep52 gives a series of parallel lines in a Zhang–Poorman plot, which is diagnostic of pure dissociative inhibition[15]. The dissociative inhibition constant (K_{id}) is 32 μM (Fig. 3A). We also used an independent biochemical method to test the ability of pep52 to disassemble the protease dimer. According to analytical gel filtration chromatography, pep52 causes the dimer to dissociate into monomers (Fig. 3B). The broad peak of the monomer indicates a lack of compact structure[41,42], which could have the therapeutic benefit of making the enzyme vulnerable to proteolysis in vivo.

The peptides identified by our approach are likely to bind specifically to HIV-1 protease. First, the ELISA and gel retardation assay for false positives demand that the peptides interact strongly with monomeric protease but not NcI. Second, the peptides do not impair the growth of *Escherichia coli* cells (data not shown), indicating a lack of affinity for critical cellular proteins and protein complexes, such as those involved in DNA replication and transcription.

Inhibitor dimers. Multivalent display could be a simple way to increase the potency of a dissociative inhibitor. Creating a homod-

Table 1. Regulatory properties of wild-type and hybrid lambda repressor proteins[a]

Plasmid	Repressor	Tetracycline susceptibility	β-Galactosidase activity (units)	Repression (%)
pSH20	None	Resistant	$(1.1 \pm 0.1) \times 10^4$	0
pSH26	NcI-PR	Sensitive	$(2.0 \pm 0.2) \times 10^3$	82
pSH27	NcI-dPR2	Sensitive	$(2.8 \pm 0.4) \times 10^2$	98
pSH28	cI	Sensitive	$(2.0 \pm 0.1) \times 10^2$	98
pSH29	NcI-zip	Sensitive	$(1.7 \pm 0.1) \times 10^3$	84

[a]NcI-PR, a fusion of cI (residues 1–131) and D25N HIV-1 protease, forms a stable dimer and functionally represses the λP_R-*lacZ*-*tet* reporter cassette in vivo. The λP_R promoter in the cassette contains only a single copy of λO_{R1} to avoid complexity arising from the cooperative binding of repressors to multiple copies of λO_R. Plasmids were transformed into *E. coli* strain MC1061 to test for in vivo binding of hybrid repressors to λO_{R1}. The low-copy-number plasmid pSH20 contains the reporter cassette alone. pSH26–29 are derivatives of pSH20 with the expression modules of various repressor hybrids driven by the *lacUV5* promoter. NcI-dPR2 is a hybrid protein of NcI and the N- and C-terminal segments of HIV-1 protease tethered by a pentapeptide linker (P^{1}Q^{2}I^{3}T^{4}L^5-GGSSG-S^{95}T^{96}L^{97}N^{98}F^{99}). NcI-zip is a hybrid of NcI and a leucine zipper domain from the yeast transcription factor GCN4. The DNA of NcI and NcI-zip are from pJH157 and pJH370 (J.C. Hu, Texas A&M University), respectively[29]. Susceptibility to tetracycline (10 μg ml^{-1}) and β-galactosidase activity were measured as indicators of binding to λO_{R1}[29,30]. Repression is calculated as follows: 1 - (β-galactosidase activity with repressor / β-galactosidase activity without repressor).

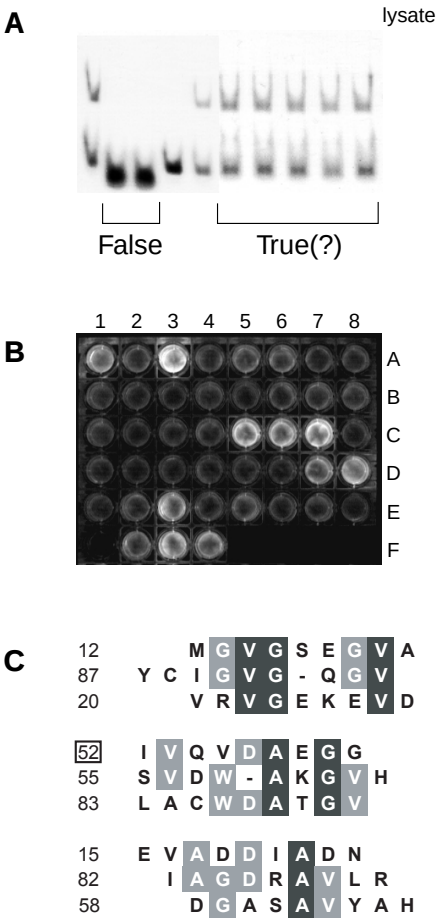

Figure 2. In vitro screens to identify true positives from the genetic selection. (A) Representative gel retardation assay. False positives that interfere with the protein–DNA interaction were identified by using a gel retardation assay to monitor the binding of cI to λO_{R1} in the presence of Trx–peptide fusions. (B) Representative high-throughput ELISA. Trx–peptide fusions not identified as false positives by the gel retardation assay were screened for binding to immobilized monomeric protease. A1 through E8 are protease-coated surfaces to which 40 lysates were applied. Binding was detected with an anti-Trx antibody. F1 is a surface coated with free cysteine to which no lysate was applied. F2 is a protease-coated surface to which a lysate containing Trx with no fused peptide was applied. F3 is a protease-coated surface to which no lysate was applied and anti-protease antibody (S. Oroszlan, National Cancer Institute) was used instead of anti-Trx antibody. F4 is a Trx-coated surface to which no lysate was applied. (C) Sequence alignment of positive isolates. The nine sequences that gave the strongest signal in the ELISA were divided into three subgroups according to their similarity. Residues that are conserved partially are in gray boxes; those conserved completely are in black boxes. The alignment was made with the MULTIALIGN interface using the BLOSOM62 algorithm[55].

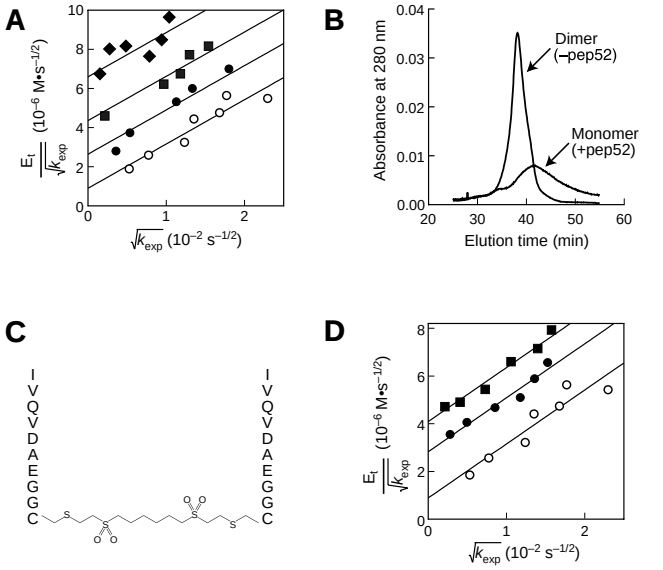

Figure 3. Analysis of inhibition of HIV-1 protease by pep52 and dim52. (A) Zhang–Poorman plot[15] for pep52. Protease activity was measured at different concentrations of pep52: 232 μM (◆), 116 μM (■), 58 μM (●), and 0 μM (○). The value of K_{id} for pep52 is (32 ± 1) μM; the value of K_d is (14 ± 3) nM. (B) Analytical gel filtration chromatogram of wild-type HIV-1 protease in the presence or absence of pep52. (C) Dim52, a dimer of pep52. (D) Zhang–Poorman plot[15] for dim52. Protease activity was measured at different concentrations of dim52: 2.96 μM (■), 1.48 μM (●), and 0 μM (○). The value of K_{id} for dim52 is (780 ± 20) nM.

imeric inhibitor by tethering the peptide in a head-to-head or a tail-to-tail fashion would present the peptide with increased valency[21,24]. Accordingly, we prepared a dimeric peptide (dim52) by adding a cysteine residue to the C terminus of pep52 and crosslinking with a homobifunctional reagent (Fig. 3C). An in vitro analysis revealed that dim52 is a potent dissociative inhibitor with a K_{id} of 780 nM (Fig. 3D), which is 40-fold less than that of the monomer. Use of an optimized crosslinker[21,23] or conformational constraint[24] could decrease K_{id} further.

AIDS chemotheraphy. Our approach is well-suited for developing new antiviral chemotherapeutic agents. Viruses have small genomes, conserving material by using multimeric proteins. Subunit interfaces in the many multimeric viral enzymes and the viral capsid are ideal targets for disruption. As an added benefit, dissociative inhibitors could intercept a viral polyprotein precursor before its processing and assembly leads to any function[20].

The high rate of viral replication and low fidelity of HIV reverse transcriptase have resulted in the emergence of HIV strains with resistance against drugs, including protease inhibitors[43,44]. To overcome resistance to individual drugs, combination therapy has been practiced in which multiple inhibitors with different antiviral mechanisms are used simultaneously to treat AIDS[13,45,46]. Recent evidence suggests, however, that the conventional triple-drug therapy can fail because of the emergence of HIV isolates with cross-resistance against different drugs[47,48]. Further, some of these cross-resistant HIV strains are also resistant to new compounds in clinical trials, including protease and reverse transcriptase inhibitors[49]. To date, all inhibitors of HIV-1 protease approved for human use are peptide mimetics that bind in the active site.

Dissociative inhibitors could be less prone to drug resistance than are active site-directed inhibitors[7]. Active site-directed inhibitors rely on a few high-affinity interactions with the protease. Protease variants with a change to a single active site residue often emerge during treatment of AIDS patients with small-molecule inhibitors and can cause drug resistance[13,45,46]. In contrast, interactions between a dissociative inhibitor and protease monomer are likely to involve many residues encompassing a large surface area. The residues in the dimeric interface are highly conserved, even among protease variants that resist active site-directed drugs[50]. The native enzyme is unlikely to tolerate changes in these residues, which are inaccessible to solvent[26].

The effect of a dissociative inhibitor can be catalytic. HIV-1 protease has several sites for autoproteolysis[51]. The presence of these sites and the absence of compact structure (Fig. 3B)[41,42] make the monomer an especially good substrate for active dimers of HIV-1 protease (as well as endogenous cellular proteases). Indeed, the asymmetry of monomer elution during gel filtration chromatography in the presence of pep52 (Fig. 3B) is consistent with its proteol-

ysis by active dimers. It is noteworthy that proteolysis not only reduces the total number of protease molecules, but also shifts the monomer–dimer equilibrium toward the inactive monomer.

Prospectus. Many biological processes rely on noncovalent interactions between proteins. The ability to interfere with specific protein–protein interactions would expedite the dissection of complex biological processes and facilitate the development of new chemotherapeutic agents. Our genetic selection (Fig. 1), along with our in vitro screens (Fig. 2), provides a powerful tool for the rapid identification of potent dissociative inhibitors in large combinatorial libraries. Here, we demonstrated the efficacy of our approach by identifying molecules that dissociate a homodimeric target protein (Fig. 3). Our approach is likewise applicable to the disruption of heterodimers, as effective hydrid repressor proteins can be assembled by heterodimerization[31,33,37]. Finally, although we panned a peptide library, our approach can also be used to sift through a library of RNA or cell-permeable small molecules for dissociative inhibitors.

Experimental protocol

Library plasmid. A degenerate oligonucleotide, 5′-AATTTA$(GGT)_3(XYZ)_9$TA-ACCCGGCG-3′, was synthesized to encode a peptide of nine random amino acid residues. The base composition of the XYZ codons, X = A(32%)/G(39%)/C(21%)/T(8%), Y = A(27%)/G(23%)/C(25%)/T(24%), and Z = G(40%)/T(60%), was as described[52]. The XYZ codons are flanked by codons for an SG_3 spacer and ochre stop codon.

The complexity of as few as six degenerate codons (4^{18}) exceeds that of the human genome. Accordingly, producing a DNA duplex in which nine codons are degenerate presents a special problem. (For example, much diversity would be lost by simply annealing two degenerate oligonucleotides.) To overcome this problem, we used the gapped-duplex method[53], in which a degenerate oligonucleotide is annealed to two shorter oligonucleotides that are complementary to nondegenerate termini. The gap in the duplex DNA is ultimately filled in vivo. Specifically, we prepared a gapped-duplex DNA by annealing the degenerate oligonucleotide with oligonucleotides 5′-ACCAC-CACCTA-3′ and 5′-TCGACGCCGGGTTA-3′. The 5′ and 3′ ends of the resulting duplex are compatible with *Eco*RI and *Xho*I cleavage sites, respectively. The primers were phosphorylated at their 5′ ends by using T4 polynucleotide kinase. An *Nde*I site was introduced at the start codon of the glutathione *S*-transferase (GST) gene in the pGEX-4T3 vector (Pharmacia, Uppsala, Sweden) by site-directed mutagenesis. The GST fragment was replaced with the *trxA* gene at the *Nde*I/*Eco*RI sites, resulting in plasmid pSH12. The phosphorylated gapped duplex was ligated to pSH12 that had been digested with *Eco*RI and *Xho*I. Electroporetic transformation of MC1061 cells with the ligated DNA yielded 5×10^8 initial transformants. The transformants were grown in Luria–Bertani (LB) medium containing ampicillin (100 μg ml^{-1}) for 4 h at 37°C before the preparation of the library plasmid (Fig. 1B). Analysis of the unamplified library indicated that 95% of clones carried inserts and that the nine XYZ codons were indeed random.

Reporter plasmid. DNA containing λP_R, λO_{R1}, and a start codon was prepared from plasmid pRZ4737 (W.S. Reznikoff, University of Wisconsin-Madison) by polymerase chain reaction (PCR) using primers as follows: 5′-CTAAGCTTGTGCGTGTTGACTATTTTACCT-3′ and 5′-AGA-GAATTCCATGGACACCTCCTTAGTACATGC-3′, and inserted into the *Eco*RI and *Hin*dIII sites of a plasmid with the p15A origin and a chloramphenicol resistance gene. A DNA fragment of transcription terminator $rrnBT_2$ was amplified from pMAL-p2 (New England Biolabs, Beverly, MA) by PCR using primers 5′-CGGTCTAGAAAAACAGAATTTGCCTGG-3′ and 5′-AAAGCG-GCAGAAACGCAAAAAGGCCAT-3′, and inserted into the *Xba*I and *Not*I sites, which are downstream of the λP_R regulatory sequences. The *lacZ* and *tet* genes were amplified from pMC1871 (Pharmacia) and pBR322, respectively, and inserted sequentially between the start codon and $rrnBT_2$ terminator using the *Nco*I and *Xba*I sites. PCR primers for the *tet* gene were designed so that the amplified gene contains the endogeneous ribosomal binding site, thereby allowing the polycistronic transcription of both *lacZ* and *tet* genes under the control of λP_R and λO_{R1}. The resulting plasmid was labeled pSH20.

A DNA fragment encoding NcI was prepared from plasmid pJH391 (J.C. Hu). A DNA fragment encoding D25N HIV-1 protease was produced by site-directed mutagenesis of plasmid pET-HIVPR (J. Tang, University of Oklahoma Health Sciences Center). From these fragments, DNA directing the expression of an NcI-PR fusion protein (*lacUV5*–NcI–PR–stop codon) was constructed and inserted into pSH20, resulting in reporter plasmid pSH26 (Fig. 1B).

Genetic selection. The library plasmid (1 μg) was transformed into electrocompetent MC1061 cells, yielding 3×10^8 transformants (or 0.06% of possible nine-residue sequences). Cells were diluted in LB containing ampicillin (100 μg ml^{-1}) and grown for 3.5 h at 37°C before being prepared for electroporation. pSH26 (1 μg) was transformed into fresh library plasmid/MC1061 electrocompetent cells. The cotransformants were plated on LB agar medium containing ampicillin (100 μg ml^{-1}), chloramphenicol (50 μg ml^{-1}), X-Gal (75 μg ml^{-1}), tetracycline (20 μg ml^{-1}), and isopropyl-β-D-thiogalactoside (IPTG; 20 μM), and incubated for 24 h at 37°C until $LacZ^+Tet^R$ colonies appeared. Library plasmids were rescued from those $LacZ^+Tet^R$ colonies.

Cell lysates for in vitro assays. Each library plasmid rescued from a $LacZ^+Tet^R$ colony was retransformed into MC1061. Each transformant was grown at 37°C in LB (2.0 ml) containing ampicillin (100 μg ml^{-1}) until log phase, when IPTG (to 0.5 mM) was added. IPTG-induced cells were grown for an additional 2.5 h and harvested by centrifugation. Cells were resuspended in 30 μl of 20 mM Tris-HCl buffer (pH 8.0) containing phenylmethyl sulfonyl fluoride (PMSF; 1 mM), dithiothreitol (DTT; 0.5 mM), ethylenediamine tetraacetate (EDTA; 1 mM), and glycerol (10 % vol/vol). Lysozyme (2,500 units; Epicentre Technologies, Madison, WI) was added to resuspended cells with brief vortexing, and the mixture was incubated at 25°C for 15 min for lysis to occur. After centrifugation at 18,000 *g* for 15 min at 4°C, the cleared lysate was recovered and kept on ice or stored frozen at -80°C.

Gel retardation assay. A fluorescein-labeled, double-stranded DNA corresponding to the λO_{R1} sequence was made by annealing oligonucleotides 5′-TTTACCTCTGGCGGTGATAG-3′ and 5′-(6-FAM)-CTATCACCGCCA-GAGGTAAA-(6-FAM)-3′, where 6-FAM is 6-carboxyfluorescein. cI protein was produced from *E. coli* strain MC1061 transformed with plasmid pFG157 (J.C. Hu). Lysates from $LacZ^+Tet^R$ cells were mixed with cI protein (30 μg ml^{-1}) and incubated for 20 min at 25°C in 10 mM Tris-HCl buffer (pH 8.0) containing $MgCl_2$ (5 mM), $CaCl_2$ (2 mM), bovine serum albumin (BSA; 50 μg ml^{-1}), sheared salmon sperm DNA (0.2 mg ml^{-1}), poly(dI-dC) (50 μg ml^{-1}), DTT (1 mM), and glycerol (5% vol/vol). Fluorescein-labeled λO_{R1} DNA was added (to 0.5 μM), and the reaction was incubated for another 15 min at 25°C before electrophoresis in a polyacrylamide (8% wt/vol) gel in 1× TBE (Tris-boric acid-EDTA) buffer at 4°C. After electrophoresis, the gel was scanned with a Fluorimager SI System (Molecular Dynamics, Sunnyvale, CA).

ELISA. HIV-1 protease was produced from *E. coli* strain BL21(DE3)pLysS transformed with plasmid pET-HIVPR (J. Tang) as described[54]. Purified protease (14 μg ml^{-1}) was incubated for 3 h in 0.10 M HEPES-NaOH buffer (pH 7.0) or in 0.10 M MES-NaOH buffer (pH 6.6) containing urea (6 M) in maleimide-activated plates (Pierce Chemical Co., Rockville, IL). Unreacted maleimide was blocked with cysteine. Unless otherwise noted, the plate was washed (5×) by mild vortexing at 25°C for 3 min with phosphate-buffered saline (PBS) between each of the following steps. Lysates from $LacZ^+Tet^R$ cells were prepared as described above, except for the use of PBS instead of Tris-HCl buffer. First, a lysate in PBS was incubated at 4°C overnight in each well. Then, anti-Trx antibody (Sigma, St. Louis, MO) in PBS containing BSA (1 mg ml^{-1}) was incubated in each well at 25°C for 1 h. Finally, anti-rabbit IgG–alkaline phosphatase conjugate (Sigma) in PBS was incubated at 25°C for 1 h. The increase in the fluorescence of FDP (Molecular Probes, Eugene, OR), a phosphatase substrate[55], was measured by scanning the plate with a Fluorimager SI System (Molecular Dynamics).

Peptide synthesis. Peptide IVQVDAEGGC (pep52) was prepared by solid-phase peptide synthesis and purified by reverse-phase high-performance liquid chromatography (RP-HPLC). The peptide and 1,6-hexane-bis-vinylsulfone (Pierce) were mixed in 0.10 M sodium borate buffer (pH 8.5) and incubated at 25°C for 3 h before quenching the reaction with cysteine. The crosslinked dimer of pep52 (dim52) was purified by RP-HPLC. The identity and purity of pep52 and dim52 were ascertained by electrospray ionization mass spectrometry with a Sciex API 365 System (Perkin Elmer) and amino acid analysis with an Amino Acid Analyzer 421 (Perkin Elmer, Foster City, CA), which was also used to determine solution concentrations.

HIV-1 protease assays. A fluorogenic substrate, R-E(EDANS)-SQNYPIVQ-K(DABCYL)-R (Molecular Probes)[56], was used to assess proteolytic activity, which was measured with a QuantaMaster1 photon-counting fluorometer (Photon Technology International, Lawrenceville, NJ) as described[57] with modifications. In the modifications, HIV-1 protease was preincubated with or without inhibitor for 30 min at 25°C in 0.10 M sodium acetate buffer (pH 4.7) containing glycerol (5% vol/vol), polyethylene glycol (PEG) 8000 (0.1% vol/vol), DTT (5 mM), EDTA (1 mM), and NaCl

(0.10 M). Substrate was added to start the reaction. Data were collected for 3–5 min to obtain the initial velocity, and treated as described[15] using eq. 1:

$$\frac{[E_t]}{\sqrt{k}} = \frac{K_m}{k_{cat}}\sqrt{k} + \frac{\sqrt{K_d K_m}}{2\sqrt{k_{cat}}} + \frac{[I]}{K_{id}} \quad (1)$$

where $[E_t]$ is the total enzyme concentration, k is the initial rate constant, [I] is the concentration of inhibitor, K_d is the equilibrium dissociation constant of the protease dimer, and K_{id} is the inhibition constant for dissociative inhibition. If inhibition is purely dissociative, then a plot of $[E_t]/\sqrt{k}$ vs $\sqrt{k}$ at different [I] consists of a series of parallel lines. Kinetic and thermodynamic parameters were obtained by nonlinear least-squares regression analysis of data with SIGMAPLOT 5.0 (SPSS).

Analytical gel filtration chromatography. Pep52 (0 or 5 mM) was incubated for 30 min at 25°C in 50 mM sodium acetate buffer (pH 4.7) containing wild-type protease (2.8 μM). The mixture was loaded onto a column (22 cm × 20 mm^2) of Superdex 75 gel filtration resin (Pharmacia) that had been pre-equilibrated with 50 mM sodium acetate buffer (pH 4.7), and eluted with the same buffer. Absorbance at 280 nm was monitored by using the program FPLC MANAGER (Pharmacia).

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