Iterative Optimization of High-Affinity Protease Inhibitors Using Phage Display. 1. Plasmin

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Received November 6, 1995; Revised Manuscript Received February 28, 1996⊗

ABSTRACT: We generated a series of libraries having variants of the first Kunitz domain of human lipoprotein-associated coagulation inhibitor (LACI-D1, also known as tissue-factor pathway inhibitor-I) displayed on bacteriophage M13 as pIII-fusions. We varied LACI-DI iteratively in two regions: the P1 region (positions 10−21) and the "second loop", (positions 31−39), which together form one end of the domain. Display-phage library Lib#1 allows 31 200 amino-acid sequences in P1 region (residues 13, 16−19). Preliminary, we screened Lib#1 against human plasmin (PLA, EC 3.4.21.7) immobilized on agarose to enrich for phage displaying variants with PLA affinity. We introduced a 1600-fold increase in second-loop diversity (residues 31, 32, 34, 39) into the population of selectants from Lib#1, yielding Lib#2. Lib#2 (allowing ∼50 million amino-acid sequences) was screened against PLA-agarose to isolate highest affinity binders. Protein EPI-P211, derived from the best isolate of Lib#2, inhibits PLA with K_i = 2 nM (at least 500-fold better than LACI-D1) and with high specificity. We used amino-acid sequences of PLA-binding selectants to design a PLA-biased library (Lib#3) which we screened against PLA. The protein EPI-P302 (derived from the best binder obtained from Lib#3) has K_i for PLA inhibition of 87 pM, which is 25-fold better than the first-round best binder and ≥12 500-fold better than LACI-D1. EPI-P302 also shows very high specificity for PLA *vs* other human proteases and is resistant to inactivation by oxidants and extremes of temperature or pH. Thus, one can use selectants from one library to design target-tailored combinatorial libraries and obtain quite stable, highly specific, very high-affinity binding molecules while maintaining an essentially human framework.

Directed evolution of binding proteins generates molecular diversity and selects binding proteins through display of proteins on genetic packages, *e.g*., phage (Ladner & Guterman, 1990). These techniques have been reviewed (Clackson & Wells, 1994; Smith & Scott, 1993).

Plasmin is a serine protease which plays a central role in hemostasis, in particular the fibrinolytic process (Colman *et al*., 1987; Robbins, 1987). Inappropriate fibrinolysis and fibrinogenolysis, leading to excessive bleeding, are frequent complications of surgical procedures that require extracorporeal circulation and are encountered in thrombolytic therapy and organ transplantation. Restoration of hemostasis requires the infusion of plasma or plasma-derived products which are costly and have associated risks for immunological reaction and exposure to blood-borne pathogens. In extreme cases antifibrinolytics, such as bovine pancreatic trypsin inhibitor (BPTI, also known as aprotinin), are administered. The aim of this study was to generate a nearly human protease inhibitor having high affinity and specifcity for plasmin. Kunitz domains are attractive for such uses (Ladner, 1996), as they are small (∼58 residues), very stable, have a 3D structure tolerant of many amino-acid sequence changes, can have new binding properties engineered (Roberts *et al*., 1992), can be produced efficiently in yeast (*e.g*., *Pichia pastoris*), and have long been used in humans (Verstraete, 1985). We picked human LACI-D1 since it is of human serum origin (minimizing immunogenic potential) and lacks glycosylation sites. Other Kunitz domains have been displayed on phage: BPTI by Roberts *et al*. (1992a,b) and Markland *et al*. (1991), and the Kunitz domain from human amyloid precursor protein-I (APP-I-D1)[1] by Dennis and Lazarus (1994a,b).

To maximize effective protein-library diversity, we pick positions to variegate from 3D structures (Figure 2) and homologous sequences (Table 1). Here we use the term "variegated" in reference to a population of DNA, of proteins, or of phage that is diversified to a greater or lesser extent at some positions (variation-tolerant residues which could influence binding) but not at other (possibly interspersed) positions (structurally crucial or buried amino acids). Thus, the diverse population is not "random" with respect to either the sites varied or the types of side groups available at each varied position. As the structure of the trypsin−BPTI complex (Brookhaven 2TPA) shows that BPTI residues 11−19, 34, and 36−39 touch trypsin,[2] changes at these residues are most likely to change binding properties of a Kunitz

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⊗ Abstract published in *Advance ACS Abstracts,* June 1, 1996.

[1] Abbreviations: APP-I-D1, Kunitz domain of amyloid precursor protein-I; BPTI, bovine pancreatic trypsin inhibitor; F.VII$_a$, Factor VII$_a$; F.XI$_a$, Factor XI$_a$; FIR, fraction of input recovered; hNE, human neutrophil elastase; LACI-D1, lipoprotein-associated coagulation inhibitor, first Kunitz domain; LB, Luria−Bertani broth; PAB, plasmin assay buffer; PEG, polyethylene glycol; pfu, plaque forming units; pKAL, human plasma kallikrein; pIII, protein III of M13; PLA, human plasmin; RSB, reaction stop buffer; RT, room temperature; TF−F.VII$_a$, complex of tissue factor and Factor VII$_a$; THBN, human thrombin; vgDNA, variegated DNA; w.t., wild-type; ::, fusion (gene or protein).

[2] Trypsin residues 39−42, 57, 58, 60, 94, 96−99, 151, 175, 189−195, 213−216, 219, 220, and 226−228 (using chymotrysinogen numbering) touch BPTI.

S0006-2960(95)02628-6 CCC: $12.00

Table 1: Frequency of Amino-Acid Types at Some Positions in 71 Natural Kunitz Domains

res. id.[a]	no. of amino-acid types[b]	contents[c]
9	15	A17 P15 I7 K6 V6 Y5 F3 R3 E2 L2 Q D S M H
9a	4	-66 G3 S P
10	8	E18 D16 V11 S6 N6 Y6 R4 A4
11*	13	T19 Q11 P9 R5 A4 S4 K4 D3 E3 V3 Y3 I2 G
12*	4	G68 D I K
13*	9	P44 R8 L7 I3 S3 N2 T2 Q V
14*	1	C71
15*	12	R27 K20 L6 F4 Y3 -3 Q2 M2 S T E N
16*	8	A41 G17 D4 K4 Q2 F R T
17*	15	M12 Y11 F9 R7 K7 H5 S4 L3 G3 N3 P2 A2 Q T I
18*	10	I37 F9 M8 V6 L3 E2 T2 A2 D K
19*	11	P16 S12 R10 K10 I7 L4 E4 T4 Q2 N F
20	7	R38 L10 A10 S8 K3 Q V
21	5	Y26 F25 W16 I2 L2
31	14	E25 A8 Q8 V8 L6 K5 R3 I2 H D T N Y Z
32	12	P19 T9 Q7 R7 E7 K6 L5 S3 A3 G2 V2 N
33	1	F71
34*	16	I19 F9 T7 V7 K5 L4 Q4 H3 D3 N3 W2 S P A R Y
35	4	Y66 W3 F S
36*	4	G57 S11 R2 T
37*	1	G71
38*	1	C71
39*	11	G30 R10 K8 Q5 M5 E4 L3 D3 P H N
40	4	G63 A6 K R
41	3	N62 K6 D3
42	12	A18 G11 R11 S8 E5 D5 N4 H3 L2 Q2 K M
44	5	N38 R24 K7 Q V
46	16	K22 E10 D8 Y8 V4 R3 P2 G2 S2 T2 L2 N2 I A Q H

[a] Residue identifier, numbering by homology to BPTI; * indicates that this residue BPTI touches trypsin in Brookhaven data set 2TPA. [b] Deletion is counted as an amino-acid type. [c] Tally of how many Kunitz domains have each amino-acid type. For example, 18 Kunitz domains have E at position 10. Single-letter codes for amino-acid types: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; −, deletion.

Table 2: Sequences of Kunitz Domains BPTI, LACI-D1, and APP-I-D1

Domain	1 5	10	15	20	25	30	35	40	45	50	55 58
BPTI	RPDFCLEPPY	TGPCKARIIR	YFYNAKAGLC	QTFVYGGCRA	KRNNFKSAED	CMRTCGGA					
LACI-D1	MHSFCAFKAD	DGPCKAIMKR	FFFNIFTRQC	EEFIYGGCEG	NQNRFESLEE	CKKMCTRD					
APP-I-D1	VREVCSEQAE	TGPCRAMISR	WYFDVTEGKC	APFFYGGCGG	NRNNFDTEEY	CMAVCGSA					

domain. Changes at most of these residues, however, are unlikely to change the 3D structure of the backbone significantly; the backbones of BPTI and APP-I-D1 are essentially identical despite 32 amino-acid differences overall and five differences in the identified contact region (Hynes *et al*., 1990). Homologous Kunitz sequences (Ladner, 1996) show which positions tolerate many amino-acid types; Table 1 shows the frequencies of amino-acid types in Kunitz domains at positions 9−21, 31−42, 44, and 46. Table 2 shows the sequences of domains BPTI, LACI-D1, and APP-I-D1. Of fourteen "BPTI-trypsin contact positions", nine show extensive variability in other Kunitz domains.

Clackson and Wells (1995) showed that a few residues dominate the binding energy of human growth hormone and its receptor; other protein−protein interactions are likely to be similar. Despite growing insight into protein−protein binding (Szardenings *et al*., 1995), it is still difficult to predict which residues will dominate high-affinity binding to a target. Thus, to obtain high-affinity (subnanomolar) binders from a Kunitz domain, we need to vary most of the residues corresponding to the trypsin-touching residues of BPTI and perhaps other nearby residues, if binding is to be through this part of the domain. We need to find most of the amino-acid changes that help binding and to remove all (or nearly all) the side groups that hinder binding. Currently, practical limitations prevent the construction of biological libraries having greater than about 10^{10} independent transformants: we cannot make a complete library in which nine positions are allowed to display 20 amino-acid types.

Wells (1990) found that effects of multiple mutations are usually roughly additive, suggesting that one can identify mutations favorable for some property and combine them to make a superior protein, as was done for human growth hormone using monovalent phage display. Dennis and Lazarus (1994a) used monovalent phage display and this approach. They isolated APP-I-D1 derivatives having affinities (K_i) for the tissue factor−Factor VII_a complex (TF−F.VII_a) between 580 and 13 nM and combined mutations to make a protein (TF7I-C) with $K_i = 1.9$ nM. The best amino acid at 39 and the best amino acid at 13 are, however, correlated. When selecting binders to a new target, one cannot know *a priori* which residue pairs may be correlated.

To avoid building huge libraries, one can screen libraries iteratively (Ladner & Guterman, 1990). A region of a displayed protein is first variegated, and the library is subjected to a preliminary screen to eliminate very bad

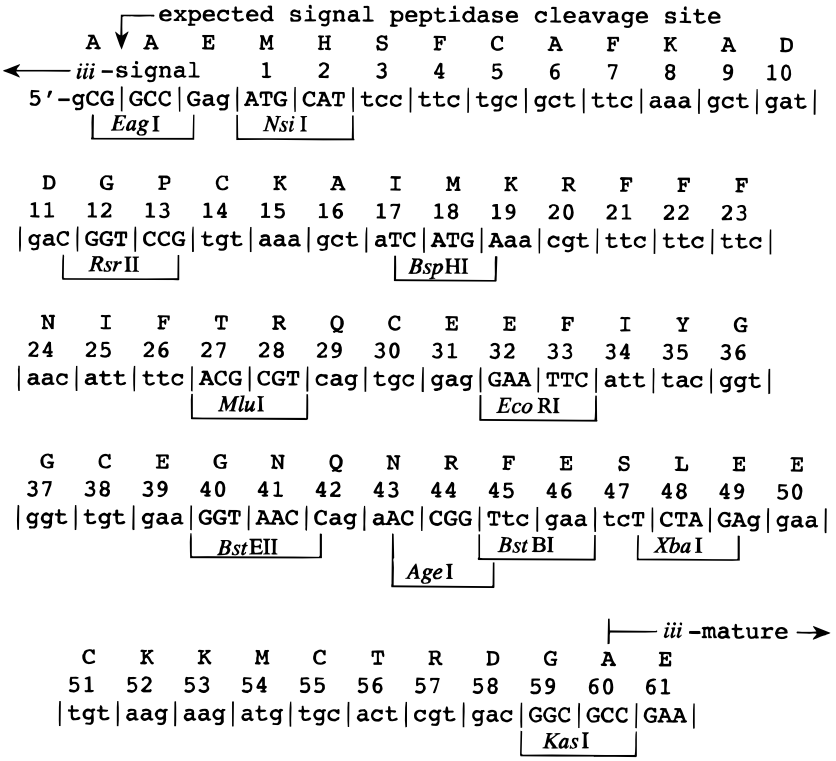

FIGURE 1: Designed *laci-d1* portion of a *iii-signal::laci-d1::iii-mature* gene showing DNA and deduced amino-acid sequence. The DNA sequence maximizes use of codons contained in *E. coli* genes known to be highly transcribed and expressed except as needed for placement of restriction sites. The restriction sites are unique to the gene and the display vector used in these studies.

binders and retain a range of good binders. When a second region is variegated in the context of selected first regions and screened stringently, crucial residues and optimally interacting pairs are identified. A very large number of proteins are allowed to be selected, but each round of variegation and screening deals with libraries of manageable size ($\sim 10^7$ or less).

EXPERIMENTAL PROCEDURES

Library Constructions. LACI-D1.III is a bacteriophage M13 derivative having gene *iii* altered by insertion of the designed DNA sequence shown in Figure 1 (Markland *et al*., 1996). All of the phage-displayed Kunitz domains we describe here have the dipeptide AE[3] fused to their amino terminus (1) to accommodate the *Eag*I restriction site and (2) to facilitate cleavage by *Escherichia coli* signal peptidase I. We confirmed the *laci-d1* DNA sequence and demonstrated functionality of the displayed LACI-D1 domain by the binding of LACI-D1.III phage to trypsin beads (Markland *et al*., 1996).

We constructed three libraries of displayed protein variants by introducing synthetic vgDNA into the *laci-d1* region (Figure 1) of LACI-D1.III. We varied two parts of the LACI-D1 amino-acid sequence shown in Figure 2: the P1 region, encompassing residues 10−21, and the "second loop", encompassing positions 31−39. Together positions 10−21 and 32−39 comprise the end of the Kunitz domain that contacts serine proteases.

To construct Lib#1 (Table 3), we ligated DNA encoding the variegation scheme Vg#1 and having *Nsi*I- and *Mlu*I-compatible ends into the *laci-d1::iii* fusion gene in LACI-D1.III. Theoretically, the 8-fold oversampling corresponds to an approximately 99.9% (=100 × [1 − exp{−5.3 × 10^5/ 6.6×10^4}]) representation of the allowed DNA sequences. The Lib#1 phage stock gave a titer of 3.6×10^{12} plaque-forming units (pfu) per mL, with each independent clone being represented, on average, 2.5×10^6 times per mL. We made Lib#2 (Table 3), ligating synthetic DNA encoding the variegation scheme Vg#2 and having *Mlu*I- and *Bst*EII-compatible ends into the RF DNA prepared from phage that had been selected from Lib#1 through two rounds for binding PLA (*vide infra*). We estimate that Lib#2, which was selected to have sequences having some affinity for PLA, contains between 10^5 and 10^6 variants. We constructed Lib#3 using Vg#3 in a manner similar to the Lib#1 construction. The Lib#3 phage stock gave a titer of 3.6×10^{11} pfu per mL, with each independent clone represented,

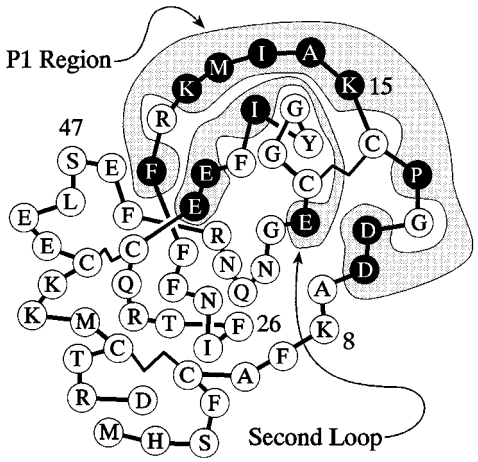

FIGURE 2: Schematic of LACI-D1 showing positions (in black) varied in present study. The P1 (positions 10−21) and second loop (positions 31−39) varied regions are enclosed. Backbone taken from Brookhaven model of BPTI 2TPA with modifications for clarity.

[3] Single-letter amino-acid codes are in Table 1.

Table 3: Libraries of LACI-D1 from Which PLA Binders Were Isolated

library name	Vg in 10−21[a]	Vg in 31−39[b]	no. of amino-acid sequences allowed[c]	no. of DNA sequences allowed	no. of transformants
Lib#1	Vg#1[d,e]	w.t.	31 200	65 536	5.3×10^5
Lib#2	Vg#1-selected for PLA binding	Vg#2[f]	5.0×10^7 [g]	2.7×10^8 [g]	5.6×10^7
Lib#3	Vg#3[h]	w.t.	5.7×10^5	1.0×10^6	7.7×10^6

[a] P1 region. [b] Second loop region. [c] Assuming that TAG is translated as Q in Sup^E strains. [d] vgDNA comprises (codons 13−19) 5′-cNt tgt aaa gSt NNt NNS NNg-3′, so that 65 636 DNA sequences encode 31 200 amino-acid sequences. [e] Mixed bases (all intended to be equimolar): N = ACGT, B = CGT, D = AGT, H = ACT, V = ACG, K = GT, M = AC, R = AG, S = CG, W = AT, Y = CT. Single-letter amino-acid codes, see Table 1. [f] vgDNA comprises (codons 31−39) 5′-Sag Sag ttc NNs tac ggt ggt tgt NNS-3′, so that 4096 DNA sequences encode 1600 amino-acid sequences. [g] Assuming complete representation of Vg#1. [h] vgDNA comprises (codons 10−21) 5′-RaS RNt ggt Nct tgt aRa gSt aNS Wtc NNS cgt tKS-3′, so that 1.0×10^6 DNA sequence encodes 5.7×10^5 amino-acid sequences.

on average, 2.3×10^5 times per mL.

Library Screening Procedures. A library screen consists of rounds in which phage are allowed to bind the target, weakly-, or non-interacting phage are removed by washes, and bound phage are then eluted. We used two screening protocols: "slow screen" and "quick screen". In a slow screen, phage from each round are amplified in *E. coli* before the next round. In a quick screen, phage recovered from the target in round N serve as input for round $N+1$ without amplification. In a quick screen, both the input and recovered number of phage decrease rapidly over several rounds, while the input level can be kept constant in a slow screen. Quick screening increases the likelihood that phage will be selected for binding rather than other irrelevant properties (*e.g.*, infectivity or growth rates).

For a slow-screen round, we combined 1×10^{10} pfu of library phage with 25 μL of PLA-agarose beads (Calbiochem, 527802) and 250 μL of phosphate-buffered saline (PBS), 1 mg of bovine serum albumin (BSA)/mL (Sigma, A-3294) in a 1.7 mL microcentrifuge tube. We incubated the phage with PLA-agarose beads on an end-over-end mixer for 2 h at room temperature (RT), pelleted the beads briefly (30 s) in a microcentrifuge, and removed the supernatant fluid containing unbound phage. To remove poorly binding phage, we washed the beads five times in 750 μL of wash buffer (PBS containing 0.1% Tween 20) using a 5 min incubation at RT per wash and then eluted remaining bound phage from the PLA-agarose beads by two consecutive 5 min incubations of the beads in 250 μL of elution buffer (150 mM NaCl, 50 mM sodium citrate, pH 2.0, 1 mg of BSA/mL). We added 50 μL of Tris·HCl pH 7.5 to each pH 2 eluate and titered the combined eluates to determine recovery. The fraction of input phage recovered (FIR) is the ratio of the total pfu in the combined pH 2 eluates to the input pfu. We used the pH-2-eluted phage to generate a new phage stock for use as input in the next round of the slow screen as described (Markland *et al*., 1996).

The quick screen protocol differs from the slow screen described above in two particulars: (1) we used between 10^{11} and 10^{12} pfu as input for the first around of the screen to compensate for anticipated losses in recovered phage over the course of the screen and (2) we used the entire neutralized pH 2 eluate (less an aliquot for quantitation of FIR) obtained from each round of the screen as the input for the next round of the screen.

Display Phage Binding Specificity. For binding studies, we purified single phage isolates from well-separated plaques and grew them using *E. coli* strain XL1-Blue (Stratagene, 200268). We used one round of the slow screen protocol to measure the relative binding affinities of individual phage isolates for the proteases PLA, human plasma kallikrein (pKAL), and human thrombin (THBN) immobilized on agarose beads. For these assays, we used commercially available PLA beads (Calbiochem, 527802) and THBN beads (Calbiochem, 605204). To prepare pKAL beads, we coupled 133 μg of pKAL (Calbiochem, 420302) to 200 μL of Reacti-Gel (6X) support (Pierce, 20259) following the manufacturer's instructions.

Production and Purification of Inhibitor Proteins. To produce small amounts of free proteins for initial analyses, we used an expression system based on the *Saccharomyces cerevisiae* matα1 promoter-signal peptide system (Miyajima *et al*., 1985). We modified the vector pMFα8 (obtained from ATCC) by insertion of synthetic DNA sequences to enable the recloning of LACI-D1 selectants, *via* unique *Eag*I and *Kas*I restriction sites, following the matα1 prepro-peptide sequence. Each expression gene had codons for the dipeptide EA fused to the 3′ end of the *laci-d1* segment to facilitate cleavage of the prepro peptide. Expression of LACI-D1 and derivatives in *S. cerevisiae* yielded 100−500 μg per 500 mL of culture after 3 day shaker-flask culture in yeast trp-selection liquid medium YND.

In our purification procedure for *S. cerevisiae*-expressed proteins, we first passed neutralized culture medium (pH 7.5) over 500 μL of trypsin-bead slurry (Pierce, 20230) in a small chromatography column and then eluted bound protein from the column using low-pH buffer (glycine/HCl, pH 1.8). We immediately neutralized column fractions with 1/10 volume of 2 M Tris·HCl (pH 8.0), analyzed the fractions for protein content and purity using SDS−PAGE, and stored combined fractions containing eluted protein in frozen aqueous solution as protein stocks. To estimate concentrations of affinity-purified protein stocks (relative to BPTI standards), we compared band intensities on silver-stained PAGE gels.

We used a *Pichia pastoris* expression system (Vedvick *et al*., 1991; Wagner *et al*., 1992) to produce larger amounts of the plasmin inhibitor protein EPI-P302.[4] To direct secretion of protein into the extracellular medium, we constructed a fusion-gene fragment having DNA encoding the *S. cerevisiae* mating factor α prepro-peptide fused to the 3′ end of *ea::laci-d1(p302)*. We incorporated the synthetic gene fragment into the plasmid pHIL-D2 (Invitrogen, San Diego, CA) between the *Bst*BI and *Eco*RI sites in the 5′ *aox1* sequence to make the expression plasmid pPIC-PLA2. Transcription of the EPI-P302 sequence is controlled by the

[4] EPI-P*j*mm comprises the AA sequence $E_{-2}A_{-1}$::LACI-D1(P*j*mm), where P*j*mm indicates the set of mutations of PLA-binding isolate mm of Lib#*j*. The residues of LACI-D1 (P*j*mm) are numbered as in Table 2.

inducible *P. pastoris aox1* promoter and the *aox1* termination and polyadenylation sequences. We incorporated pPIC-PLA2 into the genome of *P. pastoris* strain GS115 by spheroplast transformation using *Sac*I-linearized plasmid. We screened isolated transformants for EPI-P302 secretion levels and picked a high-yield isolate (PEY-73) as production strain.

To produce EPI-P302, we grew PEY-73 cultures for 3 days at 30 °C with agitation in buffered minimal methanol−complex medium (Barr *et al*., 1992) at pH 3.0 supplemented daily with 1% (v/v) MeOH. We harvested the PEY-73 cultures by centrifugation at 8000*g* for 15 min, discarded the cell pellets, and microfiltered the supernatant three times through 0.45 μ and twice through 0.2 μ filters (Costar Corporation, Cambridge, MA). We next performed a 30 K ultrafiltration on the 0.2 μ microfiltrate using a Minitan apparatus with four 30 000 NMWL filter plates (PLTK0MP04, Millipore Corporation, Bedford, MA) and then concentrated the 30 K ultrafiltrate with change of buffer (to 10 mM sodium citrate, pH 3.5) using the Minitan apparatus equipped with four 5000 NMWL filter plates (PLKCC0MP04, Millipore Corporation, Bedford, MA). In the final purification step, we applied 5 K ultrafiltration retentate to a 1 mL bed volume MP-50S (Bio-Rad Laboratories, Hercules, CA) ion exchange column and eluted the column with a pH step gradient between pH 3.0 and 7.0 using 10.0 mL volumes of 50 mM ammonium acetate in steps of 0.5 pH unit. EPI-P302 eluted from the column in the pH 6.0 fraction. We removed the volatile buffer components from the ion exchange-purified EPI-P302 by lyophilization and dissolved the dried protein in water at a concentration of 2.6 mg/mL (*ca*. 360 μM). This final protein stock was >95% EPI-P302 as indicated by inspection of silver-stained PAGE gels, and the overall yield of the purification procedure was *ca*. 40%.

K_i and k_{on} Determinations. We used a room temperature substrate-hydrolysis assay to determine apparent equilibrium dissociation constants (K_i) for inhibition of plasmin by purified inhibitor proteins. For these measurements, we first established the concentration of plasmin active sites in the PLA (Calbiochem, 527624) stock by titrating the enzyme using purified bovine aprotinin (Miles, 96-070) under conditions ([PLA] $\gg K_i$) where aprotinin functions as an effectively irreversible inhibitor.

We determined K_i for purified inhibitor proteins from measurements of residual PLA activity remaining after incubations in the presence of the inhibitors. "Percent residual plasmin activity" is the ratio of the relative substrate hydrolytic activity obtained after incubation with the inhibitor to the relative activity obtained in the absence of inhibitor, expressed as a percentage.

We fit the data to the standard bimolecular mass-action equation [*e.g.*, as given in Dennis and Lazarus (1994a)]. The "apparent" K_i measured in this way is essentially identical to the "K_i" for two reasons: substrate concentration is low (*ca*. $0.14K_M$), so that the correction is at most $(1 + 0.14)^{-1} = 0.88$, and PLA and the inhibitor are first allowed to equilibrate, and then substrate is added to this equilibrium mixture.

To determine the kinetic on rate (k_{on}) for PLA inhibition by EPI-P302, we added inhibitor to a solution already containing PLA and substrate and followed the decrease in the rate of substrate hydrolysis with time. From plots of percent residual activity *versus* time after addition of inhibitor, we fit the data to the differential equation $d[E_{free}]/dt = -k_{on}[E_{free}][I_{free}] + (K_D k_{on})[E\text{-}I]$. The value of k_{on} was adjusted to give the least-squares best fit.

Specificity Assays. We determined the specificity of soluble protein EPI-P302 against a range of human serine proteases in assays similar to that used to determine K_i for PLA. The proteases tested and the reaction conditions were as follows: pKAL (American Diagnostica, 473) 12.9 nM, substrate (Novabiochem, 03-37-1547) 17 μM, in 150 mM NaCl, 20 mM Tris·HCl, pH 7.5, 1.0 mM EDTA, 0.1% PEG (Sigma P-5413); human neutrophil elastase (hNE, Calbiochem, 324681) 8.5 nM, substrate (Sigma M-9771) 250 μM, in 150 mM NaCl, 50 mM Tris·HCl, pH 8.0, 1 mM $CaCl_2$, 0.25% Triton X-100; human plasma coagulation Factor X_a (Calbiochem, 233526) 11 nM, substrate (Novabiochem, 03-32-1517) 50 μM, in a plasmin assay buffer (PAB) containing 50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) PEG, 0.05% (v/v) Triton X-100; human pancreatic chymotrypsin (Calbiochem, 230900) 20 nM, substrate (Novabiochem, 03-32-1527) 1 mM, in 100 mM NaCl, 50 mM Tris·HCl pH 7.5, 5 mM $CaCl_2$, 0.1% PEG (Sigma, P-5413), 0.05% Triton X-100; low molecular weight human urokinase (American Diagnostica, 125) 71 nM, substrate (Novabiochem, 03-32-1515) 500 μM, in 100 mM NaCl, 50 mM Tris·HCl, pH 7.5, 5 mM $CaCl_2$, 0.1% PEG (Sigma, P-5413), 0.05% Triton X-100; human serum thrombin (Calbiochem, 605195) 0.54 nM, substrate (Sigma B9385) 10 μM, in PAB.

Stability Assays. We used measurements of residual PLA-inhibition activity to determine the resistance of EPI-P302 to inactivation by various treatments. Following treatment, we diluted the treated protein solution and used the inhibitor in the PLA-inhibition assay described above at an inhibitor:PLA ratio of 0.6. We determined functionally active inhibitor concentration from residual PLA activity and calculated percent residual inhibitor activity relative to untreated inhibitor.

For measurements of pH stability, we incubated 260 nM EPI-P302 at 37 °C for 18 h in buffers of defined pH prepared as described (Stoll & Blanchard, 1990) with the exception that all buffers contained 0.1% Triton X-100. pH range and buffers were as follows: pH less than 3.0, glycine-HCl; pH 3.0−6.5, citrate-phosphate; pH 7.0−8.0, phosphate, pH 8.0−10.0, glycine-NaOH; pH greater than 10, NaOH. For measurements of temperature inactivation of EPI-P302, we diluted the protein in 150 mM NaCl, 50 mM Tris·HCl, pH 8.0, 1 mM $CaCl_2$, 0.25% Triton X-100 to a concentration of 260 nM and then incubated the protein solutions for 30 min in water baths at desired temperatures prior to assays of residual inhibitory activities.

We tested the resistance of EPI-P302 to oxidative inactivation in 50 mM phosphate buffer at pH 7.0. The reactions contained 10 μM inhibitor in the presence of increasing amounts of the oxidant chloramine T at oxidant to protein ratios between 0 and 50. After a 30 min incubation at RT, we quenched the unreacted oxidant by adding methionine to 10 mM and assayed residual PLA-inhibitory activity in the chloramine T-treated solution.

RESULTS

Screening of Lib#1 and Lib#2 against PLA. The first iteration of library construction and screening was performed in two steps. We first constructed Lib#1 with variation restricted to positions 13 and 16−19 in the P1 region. After

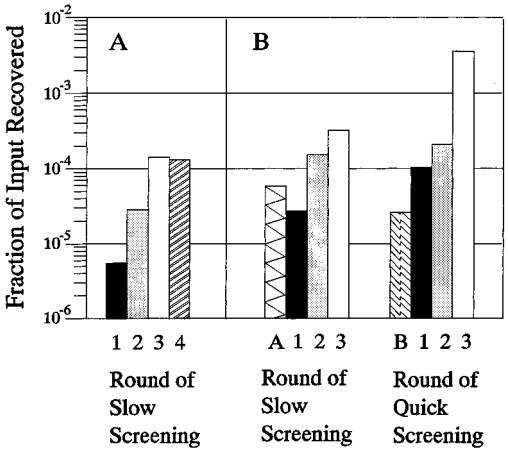

FIGURE 3: Screening of Lib#1 and Lib#2 display phage against PLA-agarose beads. Panel A shows the fraction of input phage recovered (FIR) over four rounds of slow screening of Lib#1 for binding to PLA-agarose beads. Panel B shows screening of Lib#2. Bar A is round 3 of Lib#1, shown as calibration of the activity of the PLA-agarose beads. Next are plotted FIR for three rounds of slow screening. Bar B is the FIR for round 1 of the slow screen of Lib#2. Next shown are FIR for three rounds of quick screening of Lib#2.

preliminary rounds of screening to enrich this population in variants with affinity for PLA, we added additional diversity to make Lib#2. We monitored progress in screening Lib#1 and Lib#2 for binding to PLA-agarose beads by determining the fraction of input phage recovered (FIR) from each round, as shown in Figure 3. FIR increased steadily through three rounds of slow screening of Lib#1 and leveled off in round 4 (Figure 3A). The FIR at rounds 3 and 4 was about 25-fold higher than at round 1. DNA sequences of phage isolated from round 3 showed one strong consensus and a second group of less-related sequences. Of 15 sequences, seven encoded one of the protein subsequences $H_{13}ckGPAS_{19}$ or $H_{13}ckGTAS_{19}$, with the former being more common. The remaining eight isolates contained DNA encoding a variety of sequences that can be written as $(L/R)_{13}c_{14}k_{15}(G/A)_{16}X_{17}X_{18}$-$(E/R)_{19}$ [c_{14} and k_{15} were not varied; R_{19}(arginine) is a minor component at position 19]. S, Y, N, and R occurred at position 17, and S, T, P and F occurred at position 18.

We constructed Lib#2 by incorporating Vg#2 into the phage population we obtained from round 2 of the Lib#1 screen. We infer that considerable diversity remained in the P1 region of this population because of (1) the observed variability found in round 3 isolates and (2) the increase in FIR between rounds 2 and 3 of the Lib#1 screen. The progress of a slow screen and a quick screen of Lib#2 against PLA-agarose beads is shown in Figure 3B. Bar A shows a remeasurement of the FIR of round 3 from Lib#1; the activity of the beads had decreased somewhat on storage. As seen with the slow screen of Lib#1, the FIR increased in each of three rounds of slow screen. DNA sequences of phage isolated from round 3 of the slow screen yielded two classes of phage, on the basis of the sequence in the P1 region. The first class, which comprised about 40% of round 3 phage, contained in a single sequence in the P1 region close to the consensus of Lib#1 round 3: $H_{13}c_{14}k_{15}G_{16}T_{17}A_{18}S_{19}-S_{23}$. We found, however, that neither clonally pure phage displaying one of these sequences nor free proteins of this class, expressed in yeast, bind PLA-agarose beads (data not shown). The second class includes the sequences P201−P209 in Table 4 and is characterized by an extremely tight consensus for the P1 region: $P_{13}c_{14}k_{15}A_{16}R_{17}F_{18}E_{19}$. Clonally pure isolates from this class of phage show strong binding to PLA-agarose beads.

We considered the possibility that the first class of phage possessed a growth advantage relative to other display phage in the population. We screened Lib#2 using a quick screen which should minimize effects on selected population due to differences in phage-growth phenotypes, since the procedure lacks a biological amplification step. We used the phage population from round 1 of the Lib#2 slow screen as the quick screen input (bar B in Figure 3B). FIR increased through three rounds of the quick screen. Because the input level is different from each quick-screen round, differences between slow and quick screen rounds are difficult to interpret.

DNA sequences of phage from the third round of the quick screen indicate that the population was similar to that obtained from round 3 of Lib#2 slow screen. About 40% of the phage contained the $H_{13}c_{14}k_{15}G_{16}T_{17}A_{18}S_{19}$ sequence in the P1 region. In addition, we found that all of the phage of this class from both slow and quick screens of Lib#2 contained the point mutation F23S.[5]

Sequences of PLA-Binding Lib#2 Selectants. About 60% of the phage isolates sequenced from the quick screen of Lib#2 gave sequences (P210−P232) that are very similar to the PLA-binding isolates from the slow screen. The deduced amino-acid sequences in the variegated region of several clones, differing by up to seven residues from LACI-D1, isolated from Lib#2 are shown in Table 4. These show tight selection: the sequence $P_{13}c_{14}k_{15}A_{16}R_{17}F_{18}E_{19}$ was present in 19 of 22 isolates. Within the second loop, there is strong selection for E_{31} and Q_{32} but no consensus at residue 34 or 39.

Protease Binding Properties of Selected Display Phage and Isolated Proteins. The display phage LACI-D1-(P211).III[6] is typical of the class of PLA-binding phage obtained from the first iteration of variegation and selection against PLA. We characterized this isolate for affinity and specificity of binding to proteases immobilized on agarose beads. Phage binding (measured as FIR) of LACI-D1.III and LACI-D1(P211).III to the three related human serum proteases PLA, pKAL, and THBN immobilized on agarose beads is compared in Figure 4. Relative to LACI-D1.III phage, the affinity for PLA of the PLA-selected phage LACI-D1(P211).III has increased markedly (50-fold) while the affinity for THBN and pKAL remained essentially unchanged and low.

We produced a small amount of the protein EPI-P211 using the *S. cerevisiae* production system and affinity purified the protein from the culture medium. The purified *S. cerevisiae*-produced EPI-P211 has potent inhibitory activity toward PLA, with $K_i = 2$ nM (Table 7). This is an enhancement of inhibition of at least 500-fold over LACI-

[5] Mutations are indicated as XnnZ, where X is the amino-acid type in the parent, nn identifies the position, and Z is the amino-acid type in the mutant. For example, K15R indicates changing lysine to arginine at position 15.

[6] LACI-D1 variants selected for binding PLA are designated P*j*mm where *j* is the library number and mm is the isolate number from that library. "Phage LACI-D1(P211).III" indicates an M13 derivative that displays the P211 derivative of LACI-D1 attached to the amino terminus of protein III.

Table 4: Partial Deduced Amino-Acid Sequences of Phage That Display LACI-D1 Derivatives That Bind PLA, Obtained from Lib#2

	position in LACI-D1[a]																			diffs		
identifier[b]	13	14	15	16	17	18	19	31	32	33	34	35	36	37	38	39	C2[c]	D1[d]	charge
LACI-D1	p	c	k	a	i	m	k	e	e	f	i	y	g	g	c	e	6	0	+1
Con2[f]	P	c	k	A	R	F	E	E	Q	f	T	y	g	g	c	G	0	6	+2
P201	-	"	"	-	-	-	-	Q	-	"	V	"	"	"	"	-	2	7	+3
P202	-	"	"	-	-	-	-	-	-	"	-	"	"	"	"	-	0	6	+2
P203	-	"	"	-	-	-	-	-	E	"	-	"	"	"	"	-	1	5	+1
P204	-	"	"	-	-	-	-	-	-	"	I	"	"	"	"	Q	2	5	+2
P205	-	"	"	-	-	-	-	-	-	"	I	"	"	"	"	-	1	5	+2
P206	-	"	"	-	-	-	-	-	-	"	I	"	"	"	"	F	2	5	+2
P207	-	"	"	-	-	-	-	Q	-	"	H	"	"	"	"	E	3	6	+3
P208	-	"	"	-	-	-	-	-	-	"	V	"	"	"	"	A	2	6	+2
P209	-	"	"	-	-	-	-	-	-	"	L	"	"	"	"	-	1	6	+2
P210	-	"	"	-	-	-	-	-	-	"	-	"	"	"	"	R	1	6	+3
P211	-	"	"	-	-	-	-	-	-	"	Y	"	"	"	"	D	2	6	+1
P212	-	"	"	-	-	-	-	-	-	"	H	"	"	"	"	D	2	6	+2
P213	-	"	"	-	-	-	-	-	-	"	D	"	"	"	"	A	2	6	+1
P214	-	"	"	-	-	-	-	Q	E	"	R	"	"	"	"	D	4	6	+2
P215	-	"	"	-	-	-	-	Q	-	"	Y	"	"	"	"	Q	3	7	+3
P216	-	"	"	-	-	-	-	-	E	"	A	"	"	"	"	-	2	5	+1
P217	-	"	"	-	-	-	-	-	-	"	V	"	"	"	"	-	1	6	+2
P218	-	"	"	-	-	-	-	-	E	"	V	"	"	"	"	-	2	5	+1
P219	L	"	"	G	-	-	Q	-	E	"	I	"	"	"	"	E	6	5	+1
P220	-	"	"	-	-	-	-	-	-	"	-	"	"	"	"	M	1	6	+2
P221	-	"	"	-	-	-	-	-	-	"	S	"	"	"	"	-	1	6	+2
P222	-	"	"	-	-	-	-	-	E	"	L	"	"	"	"	L	3	5	+1
P223	-	"	"	-	-	-	-	-	-	"	S	"	"	"	"	Q	2	6	+2
P224	-	"	"	-	-	-	-	-	-	"	A	"	"	"	"	A	2	6	+2
P225	-	"	"	-	-	-	-	-	-	"	I	"	"	"	"	V	2	5	+2
P226	-	"	"	-	-	-	-	-	E	"	S	"	"	"	"	K	3	5	+2
P227	-	"	"	-	-	-	-	-	E	"	V	"	"	"	"	K	3	5	+2
P228	-	"	"	-	S	-	-	-	-	"	-	"	"	"	"	N	2	6	+1
P229	-	"	"	-	S	-	-	-	E	"	-	"	"	"	"	L	3	5	0
P230	-	"	"	-	-	-	-	-	-	"	F	"	"	"	"	H	2	6	+3
P231	-	"	"	-	-	-	-	-	-	"	-	"	"	"	"	-	0	6	+2
P232	-	"	"	-	-	-	-	-	-	"	-	"	"	"	"	M	1	6	+2

[a] Numbering by homology to BPTI. Residues not shown are LACI-D1 w.t. Residues shown as " " were not varied. Residues shown as "−" were varied and the consensus (Con2) amino acid was selected. Isolates P201−P209 were obtained from slow screen round 3, and isolates P210−P232 were obtained from quick screen round 3 (Figure 3b). [b] Sequences of phage isolated for binding to PLA are designated P*j*mm, where *j* is the library number and mm is the isolated number. For example, P205 is the fifth isolate from Lib#2. [c] Number of amino-acid differences from Con2. [d] Number of amino-acid differences from LACI-D1. [e] Charge calculated as number of K, R, and H residues minus number of D and E residues. [f] Consensus of P201−P232.

D1 and is comparable to that of BPTI. The K_i between LACI-D1 and PLA is greater than 270 nM (data not shown).

Iteration of Variegation and Selection. We used the information on PLA-binding determinants (see Discussion) that we obtained from our analysis of Lib#1 and Lib#2 selectants to design Vg#3 and constructed Lib#3 as described above. We screened Lib#3 against PLA-agarose beads using a three-round quick screen with results similar to those for

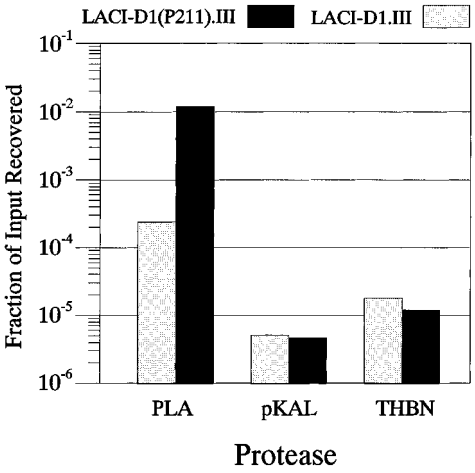

FIGURE 4: Specificity of display phage for agarose-immobilized proteases. Fraction of input recovered for LACI-D1.III (open bars) or LACI-D1(P211).III (solid bars) phage from PLA-agarose, pKAL-agarose, or THBN-agarose.

the Lib#2 quick screen. FIR increased roughly 5-fold at each round so that the FIR of around 3 (2.3×10^{-2}) was 23-fold greater than the FIR of round 1.

Sequences and PLA Binding of Lib#3 PLA-Binding Selectants. Round 3 selectants from Lib#3 are shown in Table 5; the consensus at varied residues is $(E/D)_{10}$, $(T/S)_{11}$, P_{13}, R_{15}, A_{16}, $(R/K)_{17}$, F_{18}, X_{19}, and $(W/F)_{21}$ (S_{11}, K_{17}, and F_{21} were minor components). We tested display phage clones that span the range of isolate sequences for binding to PLA-agarose beads by single rounds of the slow screen procedure. The ranking of binding of phage to PLA is shown in Table 6; BPTI.III is an M13 derivative displaying BPTI on gene III protein (Roberts *et al*., 1992a). Preliminary analysis of PLA inhibition by purified yeast-expressed proteins gave the following ranking of PLA inhibitory activity: EPI-P302 > BPTI > EPI-P315 > EPI-P211 (best from Lib#2) > EPI-P332 ≫ LACI-D1. EPI-P302 was the most potent PLA inhibitor of proteins we tested. We determined that the *S. cerevisiae*-produced protein inhibited free PLA with a $K_i \approx$ 0.09 nM. This represents about a 25-fold improvement in affinity over that of EPI-P211.

Characterization of EPI-P302. We characterized purified EPI-P302 (produced from *P. pastoris*) for PLA inhibition properties, specificity, and stability. Figure 5A shows a determination of K_i for EPI-P302 inhibiting PLA; the least-squares best K_i is 87 pM. From the rate-of-binding data (Figure 5B), we obtain $k_{on} = 0.65 \times 10^6$/M/s. We calculate $k_{off} = (K_i k_{on}) = 55 \times 10^{-6}$/s.

To determine the specificity of EPI-P302 with respect to several proteases, residual activity of various proteases was measured for increasing amounts of EPI-P302. Measurements were carried out for the proteases PLA (1.7), pKAL (12.9), hNE (8.5), Factor X_a (11), human chymotrypsin (20), urokinase (71), and THBN (0.54) (numbers in parentheses are the enzyme concentration in nM). For all of the enzymes except PLA, $IC_{50} \gg$ [enzyme], conditions where $IC_{50} = K_i$. The K_i for EPI-P302 inhibition of pKAL (~250 nM) is ~2800-fold higher than the K_i for PLA inhibition. All other proteases tested have much higher K_i values: hNE (K_i = ~800 nM); Factor X_a (K_i = ~1.3 μM); human chymotrypsin (K_i = ~10 μM); urokinase (K_i > 100 μM). For THBN, there was no inhibition ($K_i \gg 100$ μM).

Table 5: Partial Deduced Amino-Acid Sequences of Phage that Display LACI-D1 Derivatives Selected for PLA Binding from Lib#3

	position in LACI-D1[a]												
identifier[b]	**1 0**	**1 1**	1 2	**1 3**	1 4	**1 5**	**1 6**	**1 7**	**1 8**	**1 9**	2 0	**2 1**	diffs[c]
LACI-K1	d	d	g	p	c	k	a	i	m	k	r	f	7
Con3[d]	E	T	g	P	c	R	A	R	F	E	r	W	0
P301	-	-	"	-	"	-	-	-	-	G	"	-	1
P302	-	-	"	-	"	-	-	-	-	D	"	-	1
P303	-	S	"	-	"	-	-	-	-	D	"	-	2
P304	D	-	"	-	"	-	G	-	-	-	"	L	3
P305	-	-	"	S	"	-	G	-	-	D	"	-	3
P306	-	V	"	-	"	-	-	S	-	P	"	-	3
P307	-	-	"	-	"	-	-	T	-	P	"	F	3
P308	-	V	"	-	"	-	-	S	-	H	"	-	3
P309	D	-	"	-	"	-	-	S	-	G	"	-	3
P310	-	-	"	-	"	-	G	M	-	P	"	-	3
P311	-	G	"	-	"	-	-	-	-	N	"	F	3
P312	D	G	"	-	"	-	-	-	-	-	"	F	3
P313	-	-	"	-	"	-	-	-	I	S	"	F	3
P314	-	G	"	-	"	-	-	K	-	-	"	F	3
P315	-	G	"	-	"	-	-	K	-	Q	"	-	3
P316	D	S	"	A	"	-	G	-	-	-	"	-	4
P317	D	S	"	-	"	-	G	-	-	-	"	F	4
P318	D	-	"	-	"	-	-	S	-	P	"	L	4
P319	-	V	"	-	"	-	-	-	I	Q	"	F	4
P320	-	S	"	-	"	-	-	K	-	A	"	F	4
P321	-	G	"	-	"	-	-	K	-	A	"	F	4
P322	D	-	"	S	"	-	-	K	I	-	"	-	4
P323	D	S	"	-	"	K	-	-	-	D	"	-	4
P324	D	G	"	-	"	K	G	-	-	-	"	-	4
P325	-	V	"	A	"	K	G	-	-	H	"	-	5
P326	D	G	"	-	"	-	-	S	-	P	"	F	5
P327	D	S	"	A	"	-	-	K	-	R	"	-	5
P328	D	S	"	A	"	-	-	M	-	H	"	F	6
P329	D	S	"	T	"	K	-	-	-	P	"	F	6
P330	-	-	"	-	"	K	G	K	I	A	"	F	6
P331	D	S	"	A	"	K	G	K	-	-	"	-	6
P332	D	G	"	-	"	K	G	K	-	P	"	F	7

[a] Position in LACI-D1 numbered by homology to BPTI. Residues that were not varied are shown as " ". Those that were varied and have the Con3 amino acid are shown as "−". [b] Named as in Table 4, note *b*. [c] Diffs is the number of differences from Con3. [d] Consensus of P301−P332 selected from Lib#3.

Figure 6 presents the results of our experiments to test the resistance of EPI-P302 to denaturation by extremes of temperature (panel A), pH (panel B), and oxidation (panel C). The PLA-inhibition activity the inhibitor is essentially

Table 6: Phage Binding of LACI-D1 Derivatives Selected from Lib#3 for PLA Binding

identifier[a]	phage binding[b]
LACI-D1	weak
BPTI	≡1
P302	3.2×
P303	2.5×
P312	2.6×
P332	2.0×
P315	1.25×
P317	0.83×
P320	0.64×
P323	0.48×
P331	0.38×
P328	0.25×
P325	0.22×
P319	0.09×

[a] Sequences of displayed Kunitz domains given in Table 2 or Table 5. [b] Phage binding is the ratio FIR_{Pjmm}/FIR_{BPTI}, where FIR are measured from a single round of slow screen against PLA-agarose beads, P*j*mm is the purified phage isolate, and BPTI indicates BPTI.III. Phage binding is thus a measure of relative affinity of a purified display-phage isolate for PLA-agarose beads.

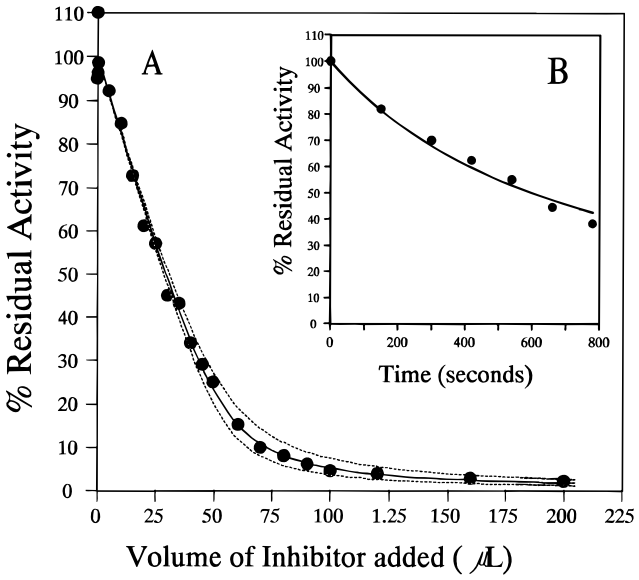

FIGURE 5: Determination of inhibition constants for EPI-P302 inhibition of human plasmin. (A) Measurement of apparent equilibrium constant for EPI-P302 inhibition of PLA. Solutions of 1.7 nM PLA and increasing concentrations of inhibitor were incubated in PAB (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 0.1% PEG, 0.05% Triton X-100) for 90 min at RT. Residual PLA activity was determined by subsequent addition of fluorogenic substrate (Sigma S-0763) at 100 μM (ca. 0.14K_M). Data are fit as described in text. Solid curve is for $K_i = 87$ pM, the least-squares best fit. Also shown are calculated curves (dotted) for $K_i = 131$ pM (upper curve, 1.5 × 87 pM) and 58 pM (lower curve, 87 pM/1.5). (B) Measurement of kinetic on rate, k_{on}, for PLA inhibition by EPI-P302. At time 0, EPI-P302 was added to a solution of PLA (1.54 nM) and fluorogenic substrate (100 nM) in 2 mL of PAB so that $I_o/E_o = 1.5$ (2.34 nM inhibitor). At times after the addition of inhibitor the percent residual PLA activity remaining in the solution was determined from measurements of sample fluorescence. The solid curve is the fit to the data as described in the text.

unaffected (retaining more than 90% of the activity of untreated material) by incubation at temperatures up to 85 °C for 30 min, 18 h incubation at pH of less than 10, and 30 min incubation in the presence of more than 50-fold molar excess of the oxidant chloramine T.

DISCUSSION

Design of Lib#1 and Lib#2. We varied the two parts of the LACI-D1 amino-acid sequence shown in Figure 2: the P1 region, encompassing residues 10−21 (because this includes the P1 residue and is expected to make many contacts to the protease), and the "second loop", encompassing positions 31−39 (because this underlies the P1 region and makes some contacts with the protease). Together positions 10−21 and 32−39 comprise the end of the Kunitz domain that could contact a serine protease in a manner homologous to BPTI-trypsin.

Not all of the residues in these regions were varied because some (*viz.*, 12, 14, 33, 37, and 38) are essentially invariant among known Kunitz domain sequences (Table 1). It is likely that at these positions the wild-type residues are required for the Kunitz domain to fold. When Dennis and Lazarus (1994a,b) varied position 12, only G was selected for binding $F.VII_a$. In natural Kunitz domains, only those having insertions or deletions nearby have a residue other than G at position 12. Dennis and Lazarus (1994b) covaried positions 14 and 38, but all selectants retained C at both positions as is found in all natural Kunitz domains.

We varied positions 13 and 16−19 first, enriched the resulting population for components that have some affinity for PLA, and then introduced variation at positions 31, 32, 34, and 39. In designing the variegation schemes, we considered the variability of Kunitz domains and the reported binding of BPTI and APP-I-D1 to PLA. BPTI is known to inhibit PLA; but published K_i values range widely, including ∼0.23−1.0 nM (Fritz & Wunderer, 1983), 0.20 nM (Schnabel *et al*., 1988), 1 nM (Wiman, 1980), 1.02 nM (Ascenzi *et al*., 1990), and 20 nM (Scott *et al*., 1987). Scott *et al*. (1987) measured K_i in very low ionic strength while the lower values were obtained under more physiological conditions. When the project started, we thought that APP-I-D1 was a potent PLA inhibitor, $K_i \approx 75$ pM (Kido, 1990). A later report of $K_i = 225$ nM (Dennis & Lazarus, 1994b) and the proteins selected in this research do not support this value. We allowed the sequence of APP-I-D1 in the 10−21 and 31−39 regions to occur, but the selected sequences are quite different from APP-I-D1.

In Vg#1, we have allowed the most common types (LPRH encoded by cNt) at position 13 (Table 1) which have very different properties. Positions 14 and 15 ("P1") are constantly C and K: Lib#1 is dedicated to K-preferring enzymes. At position 16, most (58/71) Kunitz domains have A or G; we allowed these types because they are small and are most likely to reduce unfavorable contacts. Dennis and Lazarus (1994a) allowed all 20 amino-acid types at 16 and selected for binding to $TF-F.VII_a$; all selectants had A_{16}. At position 17, the allowed set of amino acids (ACDFGHILNPRSTVY) includes the LACI-D1 wild-type (w.t.) I (seen also in BPTI) but not M (see in APP-I-D1). At position 19, the allowed set of amino acids (AEGKLMPQRSTVW) includes K (w.t. in LACI-D1) and S (w.t. in APP-I-D1) but not I (w.t. in BPTI).

At positions 31 and 32 (both tolerant of many amino-acid types), Vg#2 allows E or Q. In BPTI, these residues do not touch trypsin, but the presence of two acidic side groups could hinder PLA binding. Thus, we allow charge interactions to be eliminated by changing E to Q (Q is common at these positions). Residues 34 and 39 (both tolerant of amino-

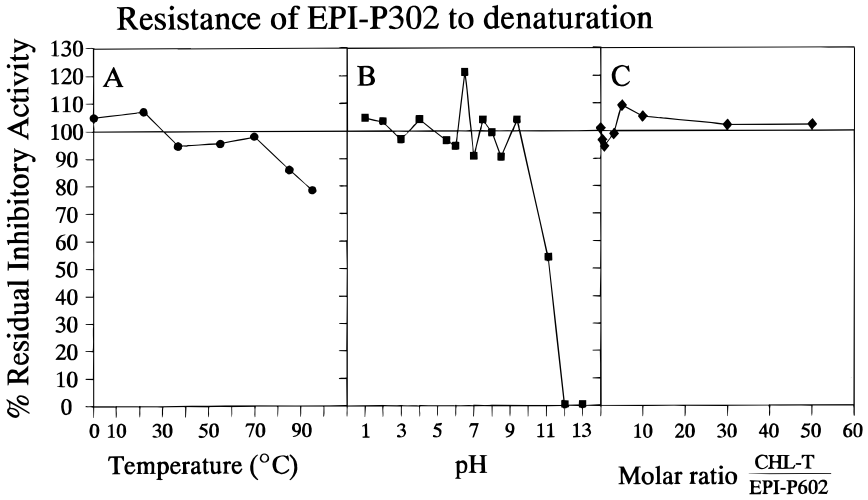

FIGURE 6: Stability of EPI-P302. Percent residual PLA-inhibition activity by EPI-P302 following various treatments is shown. Panel A shows percent residual inhibition activity (measured at RT) of EPI-P302 after incubation for 30 min at various temperatures up to 85 °C. Panel B shows percent residual inhibition activity (at RT) of EPI-P302 after incubation for 18 h at 37 °C at various pHs. Panel C shows percent residual inhibition activity of EPI-P302 after incubation for 30 min at RT with various amounts of chloramine T. Experimental details are as described in the text.

acid changes) could directly contact the target, so all amino acids were allowed. DNA sequences of isolates from the third round of Lib#1 against PLA (Figure 3A) showed significant sequence variation, and we infer that greater variation was present in the round 2 population.

Lib#2 Selectants. One class of selectants from Lib#2 yields amino-acid sequences characterized by the sequence H_{13}ckGTAS$_{19}-S_{23}$ (or similar). Neither the purified clonally pure phage isolates that display such protein variants nor the isolated proteins themselves exhibit binding to PLA immobilized on agarose beads. S_{23} is not seen in natural Kunitz domains; only Y and F are seen here. We did not find this mutation in any other phage from Lib#2, and it is possible that this mutation is needed for the binding property that allows these phage to be selected. Our finding that H_{13}-ckGTAS$-S_{23}$ phage persist at about 40% through both slow and quick screens but do not take over in a slow screen argues against their having a simple growth or infectivity advantage. When Lib#1 and derivative libraries were screened for binding to THBN and pKAL, no selectants that lack affinity for the target were found. These observations suggest that the binding site for H_{13}ckGTAS$-S_{23}$ phage involves both PLA and the LACI-D1 derivatives having P_{13}-ckARFE$_{19}$ (or similar).

The PLA-binding selectants from Lib#2 show various levels of selection at the varied positions. At positions 13, 16−19, 31, and 32 the selection is quite strong and may indicate that these side groups contribute positively to the binding. At positions 34 and 39, the selection is weaker and may show only that certain amino-acid types are incompatible with high-affinity binding.

At position 34, T, V, and I (each having side groups that branch at C_β) are the most common types and account for 18/32 of the sequences; T is most common. D, F, and R occur once each. No C, E, G, K, M, N, P, Q, or W is seen at position 34; C, E, G, and M have not been seen here in natural Kunitz domains. The branched-C_β amino acids may contribute to binding.

At position 39, no C, I, P, S, T, W, or Y is observed in Lib#2 selectants; the amino acids A, C, F, I, S, T, V, W, and Y have not been seen here in natural Kunitz domains. G accounts for 10/32 selectants, but the scatter of the other types shows that this position is not very sensitive to positive selection for PLA binding.

Design of Lib#3. We used information in the amino-acid sequences selected from Lib#2 in the design of Vg#3 which variegates the P1 region. Vg#3 was designed with three ideas in mind: (a) limiting variability at strongly selected residues increases the likelihood that new variants will bind the same site on PLA as did the isolates from Lib#2 and reduces the library size, (b) allowing variation at new positions may generate better binding, and (c) specifically excluding the $H_{13}c_{14}k_{15}G_{16}T_{17}A_{18}S_{19}$ sequence from the library. The consensus of Lib#2 PLA-binding selectants: $P_{13}-A_{16}R_{17}F_{18}E_{19}-E_{31}Q_{32}$ matches BPTI at positions 13, 16, and 17 and APP-I-D1 at positions 13 and 16. Vg#3 allows variation at positions 10, 11, 15, and 21, which were fixed in Vg#1. LACI-D1 has $D_{10}-D_{11}$ while BPTI has $Y_{10}-T_{11}$ and APP-1-D1 has $E_{10}-T_{11}$. Although residue 10 may not touch the target, charged group could act at longer range to affect binding. Thus, we allowed residue 10 to be acidic (D, E), neutral (N), or basic (K). D, E, and N occur at position 10 in natural Kunitz domains. At position 11, we allowed small (G, A), acid (D), neutral hydrophilic (S, T, N), and aliphatic (V, I) types. Of these, V and N have not been observed at position 11 in natural Kunitz domains. At position 13, P had been strongly selected from [P, H, R, L] and the 2 nM binding of EPI-P211 shows that P is compatible with good binding, so we now tested a different set (SPTA) including P; only A has not been observed at position 13. Nonbasic amino acids at position 15 are unlikely to give high-affinity PLA binding, so we allowed only K or R. Limitation of variability at position 15 helps to keep the library "pointed at" the active site; Dennis and Lazarus (1994a) also restricted the amino-acid menu at position 15. At position 16 we continued to allow G and A.

Even though R was strongly selected at position 17 (matching BPTI), we allowed the seven amino acids (IMT-NKSR). M and K had not been allowed at position 17 in Vg#1. Our reasoning was as follows. APP-I-D1 has M_{17} and was reported to bind PLA better than BPTI. If M and R could give high-affinity binding, then added diversity

should be tested. At position 18, we allowed F and I since F had been strongly selected but both BPTI and APP-I-D1 have I_{18}. At 19, we allowed 20 amino acids. APP-I-D1 has W at 21, so we allowed (FWCL).

Vg#3 allows the APP-I-D1 sequence at residues 10−21 and the BPTI sequence at residues 11−20. Note that the variegation scheme does not allow the occurrence of H_{13} or A_{18} and so should exclude the class of sequences with poor PLA-binding properties obtained from Lib#2.

Lib#3 Selectants. Not surprisingly, the larger the library the more varied the selectants. However, the selectants from Lib#3 share strong similarities both to each other and the PLA-binding selectants from Lib#2. The sequences in Table 5 are presented in order of increasing divergence from the consensus. Note that in 32 sequences there is no example of the consensus sequence and no absolute repeats; however, all are similar. At position 10, and acidic side group is selected (like APP-I-D1 and unlike BPTI). Since position 10 is probably at the edge of the interface, the selection of an acidic side group is probably for a positive contribution to binding rather than avoiding an unfavorable contact. In contrast, at position 11 an acidic amino acid does not occur. T_{11} is most common matching APP-I and BPTI.

The selection of R over K at 15 is consistent with data for BPTI and BPTI(K15R) (Scott *et al*., 1987; Fritz & Wunderer, 1983; Wiman, 1980), and with some data on APP-I-D1 variants having K_{15} (Dennis & Lazarus, 1994b) (*vide infra*). F_{18} is very strongly selected over I_{18}. Although there is no consensus at position 19, there are no examples of the Vg#3-allowed residues C, F, I, K, L, M, T, V, W, or Y. E and D are found at position 19 in 12/32 isolates (consistent with nearly unanimous selection of E_{19} from Lib#2) while a basic group occurs only once. Thus, X_{19} in the Lib#3 selected consensus means roughly: acidic (best), small hydrophilic, or P, but not large aromatic (F, W, Y), basic (K or R), or aliphatic (L, I, M, V).

Ranking of Lib#3 Selectants. The rankings of display-phage binding to PLA beads and PLA inhibitory activity of derived proteins were not identical, but the trend was similar. The best binder on phage is an extremely potent PLA inhibitor as a free protein. Discrepancies may result from uncertainties in free EPI-protein concentrations or (more likely) from the effects of the large phage particles on the kinetic properties of displayed proteins.

EPI-P302 PLA Affinity, Kinetics, and Specificity. Among the Lib#3 selectants we analyzed in display phage and free protein assays, EPI-P302 appeared to merit further study as a potential therapeutic or imaging agent. We used the *P. pastoris* expression system to produce greater amounts of the protein for subsequent analysis. Our measurements using the *P. pastoris*-produced purified protein confirmed our preliminary conclusions, based on the *S. cerevisiae*-produced protein, that EPI-P302 is a potent inhibitor of PLA. The solid curve in Figure 5A represents the best fit, $K_i = 87$ pM. The two dashed curves in Figure 5A are calculated with K_i values that differ from the best by factors of 1.5. The upper curve corresponds to $K_i = 131$ pM, and the lower curve corresponds to $K_i = 58$ pM. The observed data are (with a few exceptions at low amounts of inhibitor) all to one side of either these lines and we estimate that the "best value" of 87 pM has a margin of error of not more than about 20%.

For EPI-P302 and PLA, $k_{on} = 0.65 \times 10^6$/M/s and $k_{off} = 5.5 \times 10^{-6}$/s. Ascenzi *et al*. (1990) report K_a and k_{on} values for BPTI with several modified forms of plasmin. For Glu^1-plasmin, they report $K_a = 9.8 \times 10^8$/M, equivalent to $K_i = 1.02$ nM, and $k_{on} = 0.16 \times 10^6$/M/s. Ascenzi *et al*. are in good agreement with Wiman (1980), who reports $K_i = 1$ nM and $k_{on} = 0.09 \times 10^6$/M/s. Thus, EPI-P302 has a 4.3-fold advantage in k_{on} and a 2.7-fold advantage in k_{off} over BPTI [using values of Ascenzi *et al*. (1990)] or a 7.2-fold advantage in k_{on} and a 1.6 advantage in k_{off} [using values of Wiman (1980)]. In either case, EPI-P302 shows improvements in both k_{on} and k_{off}.

EPI-P302 is a highly specific PLA inhibitor. We did not select against binding to any protease while selecting for binding to PLA [in the way that Dennis and Lazarus (1994b) selected against binding to $F.XI_a$, PLA, and pKAL while selecting for binding to $TF-F.VII_a$]. The enzymes $F.XI_a$, $F.VII_a$, PLA, pKAL, and THBN all cleave substrates containing K or R at P1 (Schechter & Berger, 1968) and have highly similar S1 specificity pockets. At the thirty protease positions corresponding to BPTI-touching residues of trypsin, PLA and pKAL differ at 12 positions while $F.VII_a$ and $F.XI_a$ differ at 11 positions. Dennis and Lazarus (1994b) selected, from a $F.VII_a$-biased library, a protein that combines 2.7 nM affinity for $TF-F.VII_a$ with 500 nM affinity for $F.XI_a$, *viz*., 200-fold discrimination. The biased library allowed C_{14} and C_{38} to change, but only w.t. emerged. No other new (relative to libraries I−III) positions were varied. None of the selectants had as much affinity for $TF-F.VII_a$ as the designed protein TF7I-C. Several of their proteins show 1000-fold discrimination between $TF-F.VII_a$ and $F.XI_a$ but with reduction in $TF-F.VII_a$ binding. PLA and pKAL are nearly as similar as are $F.VII_a$ and $F.XI_a$. Without using pKAL for counterselection, we obtained EPI-P302 which shows a 25-fold improvement in binding over EPI-P211 and about 2800-fold discrimination between PLA and pKAL. It is likely that the high degree of discrimination is due in large part to the very high PLA affinity. The features that confer very high PLA affinity on EPI-P302 do not increase the binding to other similar proteases.

EPI-P302 Stability. EPI-P302 retains the high degree of stability characteristic of Kunitz domain proteins. Although EPI-P302 is stable over a wide range of pH, it is not so pH-stable as is BPTI (data not shown). The PLA-inhibiting activity of EPI-P302 is highly resistant to oxidants even though the molecule contains two methionines (M_1 and M_{54}). We presume that these are oxidized at least to the sulfoxide (Concetti *et al*., 1989), but this does not change the PLA binding because they are far from the binding interface. The combination of small size, high affinity, specificity, and stability make EPI-P302 an attractive candidate for development as a therapeutic or imaging agent.

Plasmin Binding Determinants. Table 7 gives partial sequences in and around the presumed PLA-contact region of domains having a range of PLA inhibitory activities. Molecules with very different charges [*viz*., BPTI, BPTI-(K15R), and EPI-P302] are potent inhibitors. Total charge does not control affinity, and this is consistent with the catalytic domain of PLA being approximately neutral. The placement and nature of charged groups appear to be important. The 25-fold improvement in affinity from EPI-P211 to EPI-P302 is probably mostly determined by differences at three positions: 11, 15, and 32. EPI-P302 has T_{11}

Table 7: Partial Amino-Acid Sequences of Kunitz Domain Proteins and Their K_i for PLA

identifier	position in Kunitz domain[a] 9	10	11	12	13	14	15	16	17	18	19	20	21	31	32	33	34	35	39	40	41	42	44	46	K_i for PLA (pm)	charge[b]
EPI-P302	a	E	T	g	P	c	R	A	R	F	D	r	W	e	e	f	i	y	e	g	n	q	r	e	87	0
BPTI(K15R)[c]	p	y	t	g	p	c	R	a	k	i	i	r	y	q	t	f	v	y	r	a	k	r	n	k	130	+6
BPTI[d]	p	y	t	g	p	c	k	a	k	i	i	r	y	q	t	f	v	y	r	a	k	r	n	k	1000	+6
IV-51C[e]	a	e	P	g	P	c	K	a	L	M	R	r	w	a	p	f	Y	y	Y	g	n	r	n	d	1700	-2
EPI-P211	a	d	d	g	P	c	k	A	R	F	E	r	f	E	Q	f	Y	y	D	g	n	q	r	e	2000	0
IV-36B[e]	a	e	P	g	P	c	K	a	L	M	K	r	w	a	p	f	V	y	Y	g	n	r	n	d	6800	-2
TF7I-C[e]	a	e	P	g	P	c	R	a	L	I	L	r	w	a	p	f	F	y	Y	g	n	r	n	d	40000	-3
IV-31B[e]	a	e	P	g	P	c	R	a	L	M	K	R	W	a	p	f	V	y	F	g	n	r	n	d	115000	-2
APP-I-D1[e]	a	e	t	g	p	c	k	a	m	i	s	r	w	a	p	f	f	y	g	g	n	r	n	d	225000	-3
EA::LACI-D1	a	d	d	g	p	c	k	a	I	m	k	r	f	e	e	f	i	y	e	g	n	q	r	e	$>10^6$	0

[a] Positions numbered by homology to BPTI. Residues shown in upper case were selected from varied pool; those in lower case were not varied. [b] Sum of K + R + H − D − E (assumes H protonated and amino and carboxy termini balance). [c] From Auerswald *et al.* (1988). [d] From Wiman (1980). Fritz and Wunderer (1983) report 230 pM; Schnabel *et al.* (1988) report 200 pM; Ascenzi *et al.* (1990) report 1.02 nM; and Scott *et al.* (1987) report 20 nM. [e] From Dennis and Lazarus (1994b).

while EPI-P211 has D_{11} (w.t.). This change is probably important because no selectant from Lib#3 has an acidic amino acid at position 11. (Note that all selectants from Lib#3 have an acidic amino acid at position 10; PLA binding of BPTI might be improved by Y10E or Y10D.) In BPTI, K15R gives only about 2-fold improvement in PLA binding (Scott *et al.*, 1987) and K15R is less strongly selected than is the elimination of D_{11}, suggesting that K15R is less important. Dennis and Lazarus (1994b) give K_i[PLA] values for APP-I-D1 and 16 variants. Remarkably, all of the variants are better PLA binders than APP-I-D1, although, for the proteins having a designation ending in "C", the experimental protocol was designed to select against PLA binding. The best PLA-binding APP-I-D1 variant is IV-51C with K_i = 1.7 nM and having K_{15}. The best PLA-binding APP-I-D1 variant having R_{15} is TF7I-C with K_i = 40 nM. On these data, one would suppose that K_{15} is superior to R_{15} in conferring PLA binding on a Kunitz domain. The optimum residue at position 15 is, however, context dependent. The context $P_{11}G_{12}P_{13}C_{14}-A_{16}L_{17}$ is superior to the context of $P_{11}G_{12}P_{13}C_{14}-A_{16}M_{17}$ in conferring PLA binding; all reported APP-I-D1 variants having L_{17} bind PLA better than any APP-I-D1 variant having M_{17}. In the context $P_{11}G_{12}P_{13}C_{14}-A_{16}L_{17}$, K_{15} gives better PLA biding than does R_{15} (IV-51C, IV-55C, IV-32B, IV-58C, IV-48C, and IV-36B *versus* IV-31B and IV-54C). In the contexts $T_{11}G_{12}P_{13}C_{14}-A_{16}R_{17}F_{18}$ and $T_{11}G_{12}P_{13}C_{14}-A_{16}R_{17}I_{18}$, the reverse is true.

EPI-P211 has Q_{32} while EPI-P302 has E_{32} (w.t.). Since Q_{32} was fairly strongly selected, we think that imposing one of E32Q or E32A on EPI-P302 could improve its PLA binding. Having an acidic amino acid at position 31 was strongly selected; thus Q31E might improve the PLA binding of BPTI. The F21W mutation carried by EPI-P302 might be important, as it considerably increases exposed hydrophobic surface. The selection of F_{18} over I_{18} suggests that PLA binding of BPTI would be improved by I18F.

Other positions that could influence binding and which show variability in natural Kunitz domains include 9, 20, 40−42, 44, and 46. Libraries in which the consensus of P1 region and second loop (Figure 2) are allowed and having variability at these additional positions could yield selectants having better binding.

CONCLUSIONS

We used relatively small phage display libraries, in an iterative process, to search a much larger sequence space involving variation at thirteen positions and selected increasingly better PLA inhibitors with each iteration. We obtained small, stable, Kunitz domains having nearly human sequences and with high affinity and specificity for PLA. These molecules are candidate therapeutics, candidate imaging agents, or lead compounds for drug development. The best inhibitor against PLA, based on the first Kunitz domain of the human LACI molecule and following two iterations of variegation and selection, has a dissociation constant of 87 pM, which is about 25-fold better than the best candidate from a single iteration and at least 12 500-fold better than the parental molecule. Further iterations might improve the affinity even further but with greater departure from the human amino-acid sequence.

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BI9526286