SUPPLEMENTAL INFORMATION

Engineered cystine knot peptides that bind αvβ3, αvβ5, and α5β1 integrins with low nanomolar affinity

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SUPPLEMENTAL METHODS

Peptide synthesis and folding

Knottin peptides were synthesized on a CS Bio CS036 instrument using 9-fluorenylmethylcarbonyl (Fmoc)-based solid phase peptide synthesis. Briefly, rink amide resin (CS Bio Company; loading 0.66 mEq/g) was swollen in *N*, *N*-dimethylformamide (DMF) for 30 min. Fmoc-protected amino acids were purchased from Novabiochem/EMD Chemicals Inc. Fmoc groups were removed with 20% piperdine in DMF. 1 mmol aliquots of amino acids were activated in a solution containing 1 mmol HOBt and 0.5 M diisopropylcarbodiimide (DIC) in DMF. After synthesis, side-chain deprotection and resin cleavage was achieved by addition of a 94:2.5:2.5:1 (v/v) mixture of trifluoroacetic acid (TFA)/trimethylsilane/ethanedithiol/water for 2 h at room temperature.

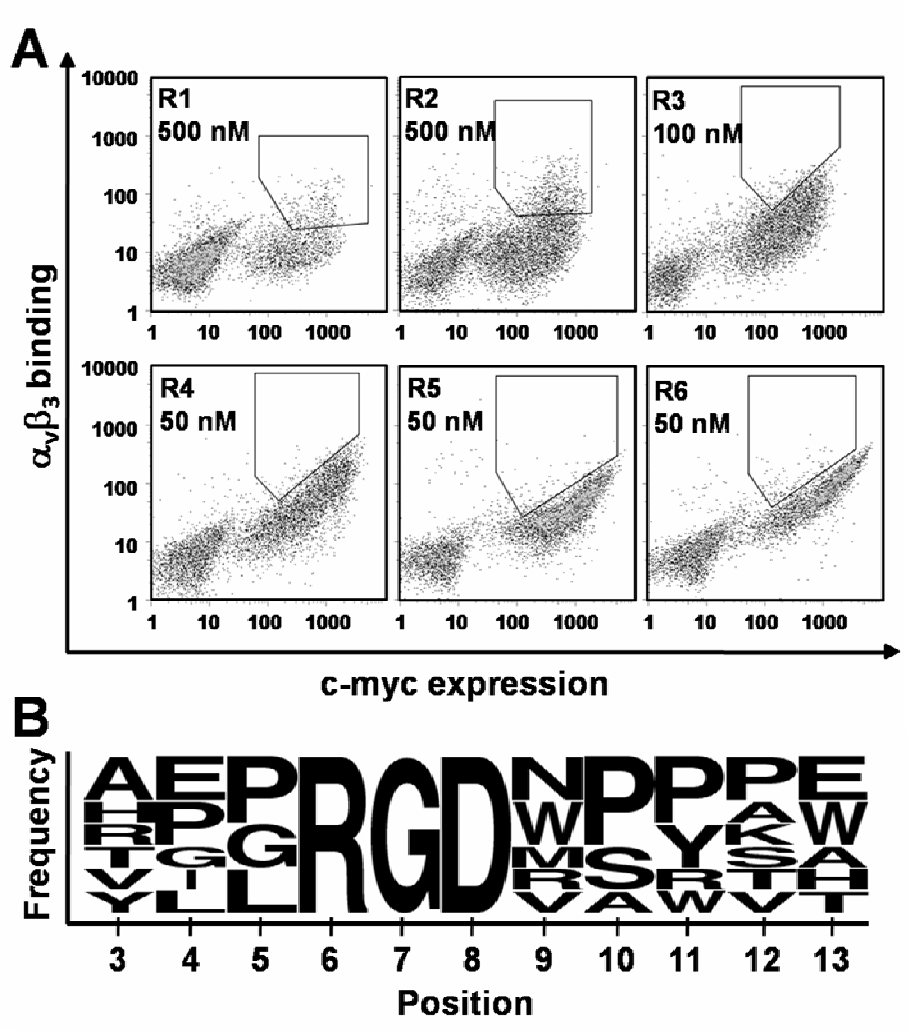
The reaction mixtures were precipitated with cold anhydrous ether, and the crude peptides were purified by preparative or semi-preparative reversed-phase HPLC using a Varian Prostar instrument and Vydac C_{18} columns. Linear gradients of 90% acetonitrile in water containing 0.1% (v/v) TFA were used for all peptide purifications, which were monitored at an absorbance of 220 nm. Peptide purity was analyzed by analytical reversed-phase HPLC using a Vydac C_{18} column. Molecular masses were determined by electrospray ionization mass spectrometry (ESI-MS) using a Waters Micromass ZQ and Waters MassLynx v4.0 or Magtran v1.02 (Zhongqi Zhang) analysis software. Representative HPLC and mass spectrometry data is shown in Supplementary Figure S2 and Supplementary Table S3.

Large scale folding reactions were performed by incubating peptides with 2.5 mM reduced glutathione and 20% dimethlysulfoxide (v/v) in 0.1 M ammonium bicarbonate, pH 9 with gentle rocking overnight. The final oxidized product was purified by semi-preparative reversed-phase HPLC as described above. Following purification, folded peptides were lyophilized and stored at room temperature until used. Purified peptides were dissolved in water, and concentrations were determined by amino acid analysis (AAA Service Laboratory, Damascus, OR). Peptide purity and molecular mass were confirmed by analytical reversed-phase HPLC and ESI-MS as described above. Representative HPLC and mass spectrometry data is shown in Supplementary Figure S2 and Supplementary Table S3.

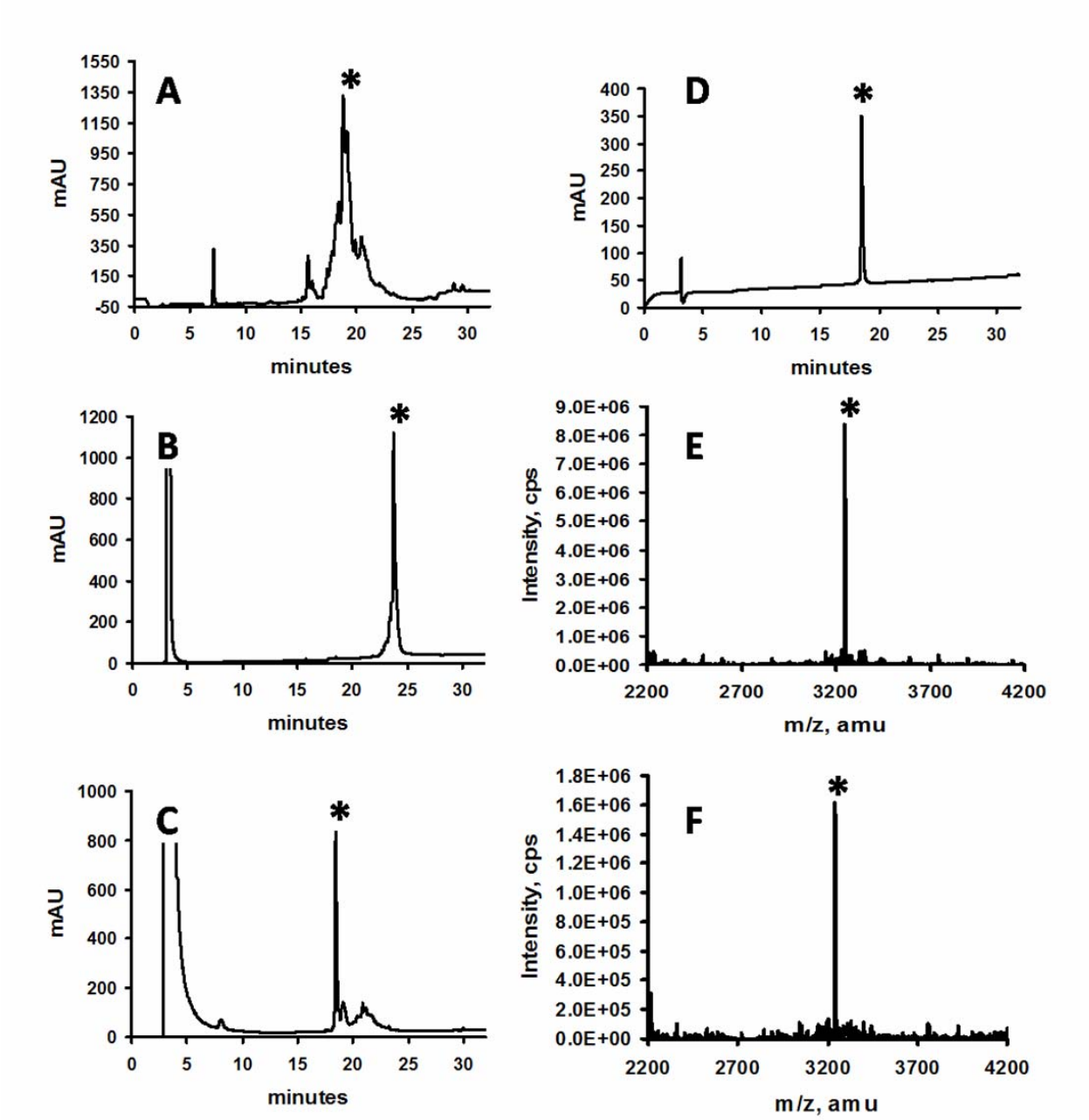
Serum stability assay

The knottin peptide 2.5D was incubated in human (Golden West Biologicals, Inc.) or mouse serum (Equitech-Bio, Inc.) at 37 ^{o}C with 5% CO_2. 50 µL aliquots were removed at various time points up to 96 h and added to 100 µL of 60% trichloroacetic acid solution to stop enzymatic activities. Following incubation on ice for 15 min, the mixture was centrifuged at 13,000 RPM for 15 min. The pellet was rinsed with 100 µL ice-cold acetone, dried, and resuspended in 500 µL of a 8M guanidinium HCl:water:acetonitrile (50:40:10) mixture. This mixture was filtered by centrifugation using a Nanosep 10K cartridge (Pall Corporation). The amount of knottin peptide in 400 µL of this mixture was analyzed by HPLC, where the area under the peak corresponding to the intact peptide was quantified at each time point.

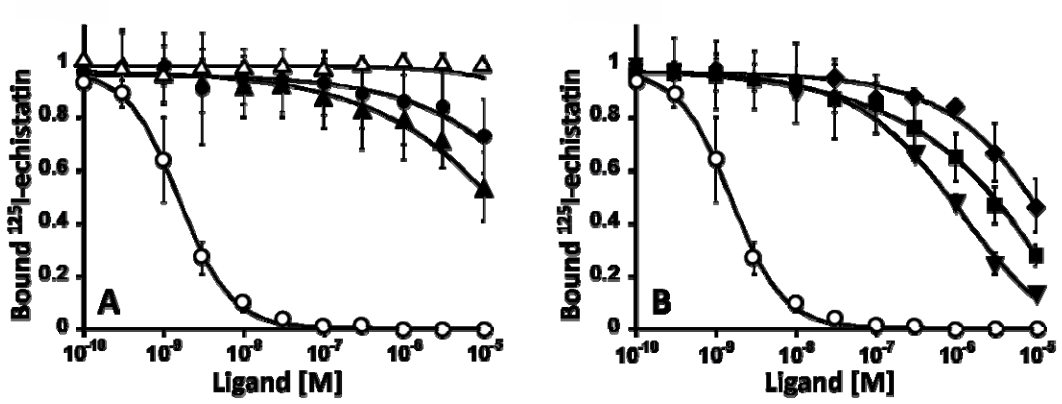
SUPPLEMENTAL FIGURES



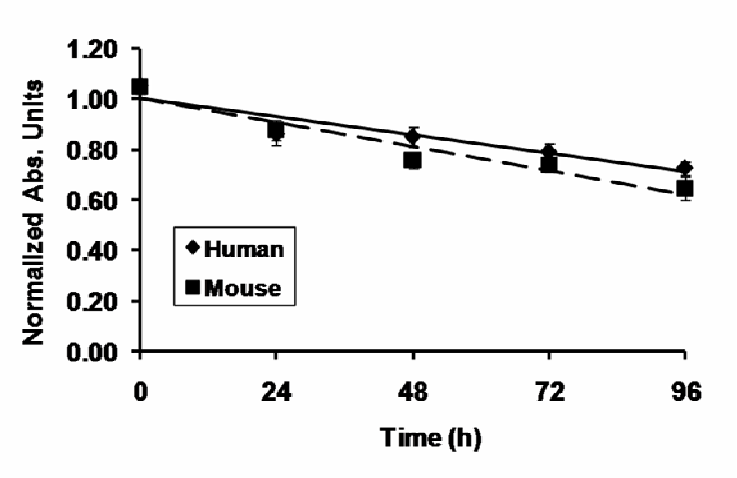
Supplementary Fig. S1. Library sort progressions and sequences from the first round of directed evolution. (**A**) Density dot plots indicating FITC (y-axis) and Alexa-555 (x-axis) fluorescence on a single-cell level. Yeast-displayed knottin peptide libraries were screened by multi-color FACS for mutants that bound the most $\alpha_v\beta_3$ integrin for a given amount of expression. The initial library contained very few of these "double positive" yeast cells, which were selected and propagated in culture for additional rounds of FACS sorting. Six rounds of FACS were used to obtain an enriched population of yeast with high $\alpha_v\beta_3$ integrin binding affinity. Integrin concentrations were reduced in successive rounds of sorting from 500 nM (rounds 1-2), to 100 nM (round 3), and 50 nM (rounds 4-6). (**B**) Sequence logo showing the relative frequencies of amino acids present in the engineered EET-II loop from 7 individual clones. The first amino acid shown is the third amino acid of the parent scaffold EETI-II. This figure was generated using online weblogo software (weblogo.berkeley.edu).



Supplementary Fig. S2. A series of reversed-phase HPLC traces and mass spectrometry data demonstrates the entire process of synthesis, purification, and folding of a representative knottin peptide, mutant 2.5D, which is indicated by asterisks. (**A**) Preparative HPLC trace of the crude solid phase peptide synthesis reaction mixture. Analytical HPLC traces of the (**B**) purified reduced/unfolded peptide, (**C**) oxidative folding reaction of the peptide, and (**D**), purified oxidized/folded peptide. (**A**) Gradient = 20 - 50% solvent B (90% acetonitrile/10% water/0.1% trifluoroacetic acid) over 14 min. (**B**-**D**) Gradient = 10 - 50% solvent B over 30 min. (**E**) Electrospray ionization mass spectrometry (ESI-MS) of the purified reduced peptide. (**F**) ESI-MS of the purified oxidized peptide.



Supplementary Fig. S3. Competition binding of peptides to surface-immobilized $\alpha_{iib}\beta_3$ integrin. To quantify $\alpha_{iib}\beta_3$ integrin binding, varying concentrations of unlabeled peptides were incubated with ^{125}I-labeled echistatin and allowed to compete for binding to microtiter plates coated with detergent-solubilized $\alpha_{iib}\beta_3$ integrin. Unbound ^{125}I-echistatin was removed, and the amount of plate-bound ^{125}I-echistatin remaining was measured. The fraction of ^{125}I-echistatin bound is plotted versus the concentration of unlabeled (**A**) c(RGDyK) (●), FN-RGD2 (▲), and FN-RDG2 (△), and (**B**) evolved knottins 1.5B (▼), 2.5D (■), and 2.5F (◆). (**A,B**) Unlabeled echistatin (○) was used as a positive control to compare binding data from different experiments. Data are representative of several experiments.



Supplementary Fig. S4. Serum stability of knottin peptides. Stability of knottin peptide 2.5D in mouse serum was measured by reversed-phase HPLC. The percentage of intact peptide (y-axis) remaining after incubation in serum at 37 °C was plotted as a function of incubation time (x-axis).

Supplementary Table SI. Sequences of clones isolated from the first and second rounds of directed evolution.

Clone	Primary Sequence
1.4A	GC**AEPRGDMPWTW**CKQDSDC**L**AGCVCGPNGFCG
1.4B	GC**VGGRGDWSPKW**CKQDSDC**P**AGCVCGPNGFCG
1.4C	GC**AELRGDRSYPE**CKQDSDC**L**AGCVCGPNGFCG
1.4E	GC**RLPRGDVPRPH**CKQDSDC**Q**AGCVCGPNGFCG
1.4H	GC**YPLRGDNPYAA**CKQDSDC**R**AGCVCGPNGFCG
1.5B	GC**TIGRGDWAPSE**CKQDSDC**L**AGCVCGPNGFCG
1.5F	GC**HPPRGDNPPVT**CKQDSDC**L**AGCVCGPNGFCG
2.3A	GC**PEPRGDNPPPS**CKQDSDC**R**AGCVCGPNGFCG
2.3B	GC**LPPRGDNPPPS**CKQDSDC**Q**AGCVCGPNGFCG
2.3C	GC**HLGRGDWAPVG**CKQDSDC**P**AGCVCGPNGFCG
2.3D	GC**NVGRGDWAPSE**CKQDSDC**P**AGCVCGPNGFCG
2.3E	GC**FPGRGDWAPSS**CKQDSDC**R**AGCVCGPNGFCG
2.3F	GC**PLPRGDNPPTE**CKQDSDC**Q**AGCVCGPNGFCG
2.3G	GC**SEARGDNPRLS**CKQDSDC**R**AGCVCGPNGFCG
2.3H	GC**LLGRGDWAPEA**CKQDSDC**R**AGCVCGPNGFCG
2.3I	GC**HVGRGDWAPLK**CKQDSDC**Q**AGCVCGPNGFCG
2.3J	GC**VRGRGDWAPPS**CKQDSDC**P**AGCVCGPNGFCG
2.4A	GC**LGGRGDWAPPA**CKQDSDC**R**AGCVCGPNGFCG
2.4C	GC**FVGRGDWAPLT**CKQDSDC**Q**AGCVCGPNGFCG
2.4D	GC**PVGRGDWSPAS**CKQDSDC**R**AGCVCGPNGFCG
2.4F	GC**YQGRGDWSPSS**CKQDSDC**P**AGCVCGPNGFCG
2.4G	GC**APGRGDWAPSE**CKQDSDC**Q**AGCVCGPNGFCG
2.4J	GC**VQGRGDWSPPS**CKQDSDC**P**AGCVCGPNGFCG
2.5A	GC**HVGRGDWAPEE**CKQDSDC**Q**AGCVCGPNGFCG
2.5C	GC**DGGRGDWAPPA**CKQDSDC**R**AGCVCGPNGFCG
2.5D	GC**PQGRGDWAPTS**CKQDSDC**R**AGCVCGPNGFCG
2.5F	GC**PRPRGDNPPLT**CKQDSDC**L**AGCVCGPNGFCG
2.5H	GC**PQGRGDWAPEW**CKQDSDC**P**AGCVCGPNGFCG
2.5J	GC**PRGRGDWSPPA**CKQDSDC**Q**AGCVCGPNGFCG

Supplementary Table SII. Degenerate codons used to construct the biased library. (http://www.sigma-genosys.com/calc/DNACalc.asp). X = Position of the residue to the left (X_{-n}) or right (X_n) of the RGD motif. Y_o = Amino acid residue 21.

Position	Codon	Encoded Amino acid(s)
X_{-3}	NHT	F,S,Y,L,P,H,I,T,N,V,A,D
X_{-2}	SNA	G,P,E,L,A,V,Q,R
X_{-1}	SSA	G,P,A,R
R	AGA	R
G	GGG	G
D	GAT	D
X_1	WRS	N,S,K,R,Y,C,S,W, stop
X_2	BCT	A,S,P
X_3	CST	P,R
X_4	NHA	S,K,V,A,E,I,L,P,Q,T,stop
X_5	DVG	E,W,T,K,A,S,G,R,stop
Y_o	CNA	L,P,Q,R

N = A+T+G+C; H = A+C+T; S = C+G; W = A+T; R = A+G; B = C+G+T
D = A+G+T; V = A+C+G

Supplementary Table SIII. Characterization of peptides by mass spectrometry. Each knottin peptide used this study has been characterized in its unfolded and folded forms by ESI-MS, where we report the neutral mass.

Peptide	Expected Neutral Mass (Da)		Observed Mass (Da)	
	Reduced	Folded	Reduced	Folded
c(RGDyK)	621.7	619.7	n/a	n/a
FN-RGD2	3110.4	3104.4	3110.3	3103.9
1.5B	3265.6	3259.6	3265.5	3259.8
2.5D	3248.6	3242.6	3248.6	3242.8
2.5F	3296.7	3290.7	3296.4	3290.3
FN-RDG2	3110.4	3104.4	3109.9	3104.5

n/a = not applicable