PROTEINS: Structure, Function, and Genetics 14:509–515 (1992)

RAPID COMMUNICATION

Identification of Novel Peptide Antagonists for GPIIb/IIIa From a Conformationally Constrained Phage Peptide Library

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ABSTRACT **Methods have recently been developed to present vast libraries of random peptides on the surface of filamentous phage. To introduce a degree of conformational constraint into random peptides, a library of hexapeptides flanked by cysteine residues (capable of forming cyclic disulfides) was constructed. This library was screened using the platelet glycoprotein, IIb/IIIa, which mediates the aggregation of platelets through binding of fibrinogen. A variety of peptides containing the sequence Arg-Gly-Asp or Lys-Gly-Asp were discovered and synthesized. The cyclic, disulfide-bonded forms of the peptides bound IIb/IIIa with dissociation constants in the nanomolar range, while reduced forms or an analogue in which Ser replaced the Cys residues bound considerably less tightly. These results demonstrate the feasibility for introducing conformational constraints into random peptide libraries and also demonstrates the potential for using phage peptide libraries to discover pharmacologically active lead compounds.**

Key words: phage peptide libraries, conformationally constrained peptides, IIb/IIIa peptide antagonists

INTRODUCTION

Drug discovery has traditionally relied on screening large numbers of organic molecules for identification of novel lead compounds. More recently, construction of large peptide libraries by either chemical[1–4] or biological means[5–8] has greatly expanded the repetoire of potential compounds for pharmaceutical leads. These libraries readily encompass $> 10^7$ different peptides thereby creating a rich source of structural diversity. To date peptide libraries have been successfully utilized for the rapid determination of epitopes to monoclonal antibodies.[1–5,7,8] Similarly, it should be possible to use these libraries to identify peptides that are able to mimic natural ligands of receptors, either as antagonists or agonists. Peptide sequences that are selected from the library by virtue of specific binding to a target can then serve as the starting point for the design and construction of peptidomimetics. Despite the promise of this technique, there have been no reports of the use of phage libraries to discover small peptides that bind specifically to cellular receptors. This presumably does not reflect a limitation of the technique as a variety of small proteins including human growth hormone[9,10] have been expressed on the surface of phage, and found to bind purified receptors in vitro. We therefore decided to screen peptide libraries using the platelet membrane protein IIb/IIa.

The glycoprotein IIb/IIIa ($\alpha_{IIb}\beta_3$) is a member of the integrin family of cell adhesion proteins that mediate platelet aggregation through the binding of fibrinogen and von Willebrand factor; antagonists of this process have been extensively pursued as potential antithrombotic agents.[11–13] Upon stimulation with a variety of agonists, the IIb/IIIa on the surface of platelets coalesces into patches and undergoes a conformational change allowing it to bind to several extracellular adhesive proteins.[11] IIb/IIIa and several other integrins recognize the common sequence motif Arg-Gly-Asp (RGD).[12–14] Synthetic peptides incorporating RGD bind IIb/IIIa competitively with respect to fibrinogen and thereby inhibit platelet aggregation.[12] The structural context of the RGD sequence determines the specificity and affinity of this interaction. For example, cyclic peptides containing the RGD sequence have higher affinity for integrins than their linear counterparts.[15] In addition, a number of snake venoms in which the RGD sequence is contained within a more structured environment, also bind with high affinity.[16]

Received July 15, 1992; accepted July 16, 1992.
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It was anticipated that a library in which peptide sequences form cyclic structures would aid in the identification of peptide sequences that bind to IIb/IIIa with high affinity. In the peptide libraries reported thus far sequence constraints have not been imposed on the random peptides; each peptide is either totally unconstrained[4] or has one free end while the other end remains tethered either to a solid support[1-3] or to a protein on the surface of a phage.[5-8] Therefore we constructed a library of random hexapeptides bounded by two Cys residues (C-X_6-C library). Given the relatively favorable oxidation potential of Cys-containing peptides with this ring size[17] we assumed that most random sequences would spontaneously oxidize in the presence of oxygen to form cyclic disulfides on the surface of phage. This expectation appeared to be a reasonable one based on the observation that several disulfide containing proteins have been expressed in native form on the surface of phage.[18-22] The cyclic form of the peptides should have restricted conformational freedom providing higher affinity and specificity for a receptor.

MATERIALS AND METHODS

Strains

Escherichia coli strains K91 (thi/HfrC)[23] was a gift from G. Smith, LE392 (el4$^-$, hsdR514, supE44, supF58, lacY, galK2, galT22, metB1, trpR55/F'kan^r) was provided by L. Enquist, and CJ236 (dut1, ung1, thi1, relA/pCJ105) was obtained from V. Nagarajan. Phage M13mp19[24] was purchased from Bethesda Research Laboratories (Bethesda, MD). Plasmid pRH43[25] was used as a source for the kanamycin resistance gene cassette.

Construction of Peptide Libraries

For the display of random peptides on the surface of phage, libraries were constructed using the gene III fusion vector M13PL-6 (Fig. 1). M13PL-6 was constructed from M13mp19[24] with the following modifications. First, the kan^r from pRH43[25] was inserted into the multiple cloning site following digestion of the phage DNA with *Eco*RI and *Bam*HI. A unique *Kpn*I site was subsequently reintroduced into the leader sequence of gIII as reported by Devlin et al.[6] by site-directed mutagenesis[26] following propagation of the phage on CJ236 to incorporate uracil into the DNA. In addition, the tyrosine codon at position 15 of the gIII signal sequence was replaced with an amber codon using the mutagenic oligonucleotide 5' CGGAGTGGGACTAGAAAGGTACC 3', and a unique *Bst*XI site was introduced at the junction between the signal sequence and the N-terminus of the mature gIII protein using the oligonucleotide 5' CTTTCAACAGTTCCAGCGGAGTGGGAATAGAAAGG 3'. The final M13PL-6 construct was propagated on LE392 in order to suppress the amber codon in the signal sequence.

Peptide libraries were constructed by cleaving M13-PL6 with *Kpn*I and *Bst*XI and ligating the vector with the appropriate oligonucleotides for either the CX_6C library or a linear hexapeptide (X_6) library (Fig. 1). Sequences encoded by the oligonucleotides for the random peptides in the C-X_6-C library were inserted three residues from the N-terminus of gpIII (Ala-Glu-Cys) since there is evidence that indicates that at least for gVIII of M13 there is inefficient cleavage by the signal peptidase when Cys is immediately adjacent to the cleavage site.[27] The X_6 library was similarly constructed with the exception that the random peptide insertion was made immediately following the signal peptidase cleavage site. The random peptide sequences were connected to gIII via two (C-X_6-C) or three (X_6) Gly residues to provide a flexible tether between the fused peptide and the gIII protein. All oligonucleotides were phosphorylated separately with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) prior to annealing. The oligonucleotides coding for the random peptide sequences (N = A,G,C,T in equimolar mix) were annealed with the two shorter complimentary oligonucleotides at a ratio of 1:100,[7] and then ligated with the vector using T4 DNA ligase (New England Biolabs) at 16°C overnight. The resulting gapped ligated DNA was electorporated into K91. Following electroporation, cells were diluted into SOB (bacto-tryptone at 20 g/liter, yeast extract at 5 g/liter, NaCl at 0.58 g/liter, KCl at 0.19 g/liter, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) medium and grown for approximately 6 hr to amplify the library. Random clones were selected from each library for DNA sequencing to ensure that there was no overall bias in the nucleotide sequence coding for the random peptide sequence, and that the majority of phage contained inserts (>90%). Because the oligonucleotides used to construct the libraries revert the amber codon in the signal sequence to the original tyrosine codon, only those phage receiving an insert should be able to grow on the nonsuppressing host K91.

Preparation of Receptor-Coated Plates

Microtiter dishes (96 well, Costar, Cambridge, MA) were coated with IIb/IIIa receptor as described.[28] The receptor solution was incubated on the plates overnight at 4°C; plates were then frozen at −70°C until use. Before incubating with the library, plates were thawed at room temperature and washed twice with 100 μl Buffer A (50 mM Tris, 150 mM NaCl, 0.5% Tween 20; 0.5 mM $CaCl_2$, pH 7.5), blocked with 100 μl 29 mg/ml BSA in 0.1 M $NaHCO_3$ for 1 hr and washed 3 more times with Buffer A.

Selection and Identification of Phage That Bind to IIb/IIIa

In a manner similar to the biopanning procedure described previously by Parmley and Smith, peptide libraries were screened for sequences that bound to

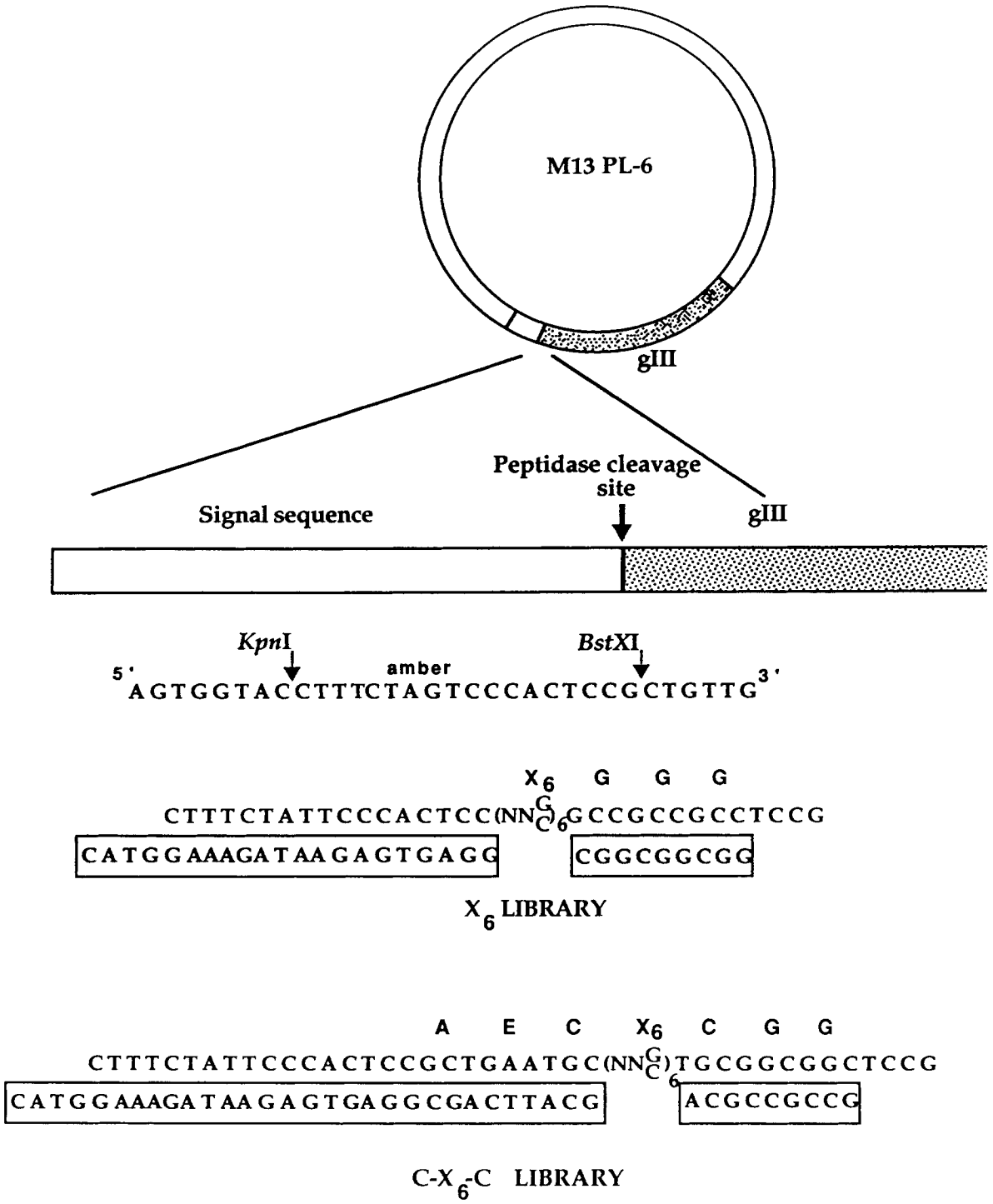

Fig. 1. Strategy for the construction of phage-peptide display libraries. Peptide libraries were constructed by digesting M13-PL6 with *Kpn*I and *Bst*XI and ligating the vector with the oligonucleotides shown for the X_6 or CX_6C libraries. The oligonucleotides coding for the random peptide sequences were annealed with the two shorter complimentary oligonucleotides (boxed sequences) prior to ligation into the appropriately digested vector.

GPIIb/IIIa.[29] From each peptide library, approximately 10^{10} phage in 100 μl were incubated per well overnight at 4°C. Nonspecifically associated phage were washed away with fifteen 100 μl washes of buffer A. In Round 1 bound phage were eluted with 100 μl of 550 μM SK&F 106760;[30] for Round 2, bound phage were eluted first with 100 μM SK&F 106760 and then with glycine-HCl (pH 2.2). Eluted

phage were titered for plaque forming units on K91. Individual plaques were isolated and used to propogate phage for preparation of single stranded DNA for sequencing.

Peptide Synthesis

Peptides were synthesized by solid phase synthesis methods on PAL resin[31] using Fmoc-OPfp amino acids with the following side chain protection scheme: Cys(Trt), Lys(Boc), and Asp(tBu). Coupling of threonine residues was accomplished using the Fmoc-Thr(tBu)-ODhbt derivative and Fmoc-Arg (Pmc) was coupled using TBTU/*N*-methylmorpholine activation.[32] The N-terminus was acetylated using acetic anhydride/pyridine. Peptides were cleaved from the resin with trifluoroacetic acid:thioanisole:ethandithiol:anisole (9:0.5:0.3:0.2) for 2 hr at room temperature and were precipitated with ether after filtering off the resin. Peptides were oxidized by stirring a 1 mg/ml solution of peptide in 10 mM ammonium acetate pH 8.5 overnight at room temperature. Peptides were purified by reversed-phase HPLC and the identity and homogeneity confirmed by amino acid analysis and Fast Atom Bombardment/Mass Spectrometry.

Platelet Aggregation and IIb/IIIa Binding Studies

Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were obtained from venous blood. Platelet aggregation was measured on a PAP-4 Platelet Aggregation Profiler using PPP as the blank (100% transmittance); unactivated PRP was used for 0% transmittance. 20 μl of a 10–100 μM agonist mixture (ADP, collagen, arachidonate, epinephrine, and thrombin) was added to 200 μl of PRP to initiate platelet aggregation. To measure inhibition of aggregation various concentrations of peptide were added to PRP prior to platelet activation and a comparison of aggregation profiles with and without inhibitor was made. IIb/IIIa binding studies were performed using a competition assay with ^{125}I-labeled fibrinogen as described.[28]

RESULTS AND DISCUSSION

Selection of GPIIb/IIIa Binding Sequences

To identify peptides that bind IIb/IIIa both the X_6 and CX_6C peptide libraries were screened with receptor that had been immobilized on microtiter dishes. Rather than elute bound phage with glycine-HCl (pH 2.2) as has been previously reported for similar experiments where a monoclonal antibody is epitope mapped,[5–8] we used a ligand specific for IIb/IIIa, SK&F 106760.[30] In so doing we anticipated that we would eliminate elution of those phage that associate nonspecifically with the receptor, which might otherwise be eluted by low pH. In fact, experiments in which low pH was used in the first elution step failed to give rise to identifiable sequence motifs, suggesting that this treatment had eluted nonspecifically absorbed phage (data not shown). Following elution with SK&F 106760, phage were recovered, amplified, and then readsorbed to immobilized receptor. In the second round phage were eluted first with SK&F 106760 and then by glycine-HCl (pH2.2). The peptide sequences encoded by the eluted phage were similar regardless of the eluting agent used in the second round. As shown in Table I, the second round of selection yielded a 500-fold increase in the percentage of phage recovered from the $C\text{-}X_6\text{-}C$ library as compared to the X_6 library. This suggests the $C\text{-}X_6\text{-}C$ library gives rise to far more clones that bound the IIb/IIIa receptor.

TABLE I. Recovery of Bound Phage From IIb/IIIa Coated Plates: CX_6C Library Shows Higher Enrichment for IIb/IIIa Binding Phage

Library	Input titer (10^{-9})	Eluted titer (10^{-3})*	% Recovery (10^{-4})
X_6			
Round 1			
	14.6	3.6	0.25
Round 2			
	3.74	0.3[a]	0.09
		0.3[b]	0.09
CX_6C			
Round 1			
	9.0	0.9	0.09
Round 2			
	1.34	69.0[a]	51.6
		40.7[b]	30.4

*In the second round of selection, bound phage were eluted with either [a] 100 μM Sk106760 or [b] glycine buffer (pH2.2).

Individual clones from the $C\text{-}X_6\text{-}C$ library were selected and the nucleotide sequence of the inserts determined. Each clone encoded an RGD-like sequence (Table II). The peptide sequences split into two predominant groups of similar sequences. The first contains RGD followed by two hydrophobic then one hydrophilic residue. Previously, hydrophobic residues C-terminal to RGD have been shown to enhance the affinity for IIb/IIIa of small synthetic peptides and small proteins isolated from venoms.[33] The second class contains three predominantly hydrophilic residues followed by an RGD or Lys-Gly-Asp (KGD) sequence. Finally, one clone encoded a sequence (Cys-Ser-Arg-Gly-Asp-Val-Pro-Cys) that was homologous to the IIb/IIIa binding segment of fibronectin (Arg-Gly-Asp-Ser-Pro-Cys).[14]

GPIIb/IIIa Binding and Antiaggregatory Activity of Synthetic Peptides

Several peptides from the two classes of were synthesized and tested for inhibition of ^{125}I-labeled fibrinogen binding to platelets and inhibition of platelet aggregation in platelet-rich plasma (PRP) after stimulation of platelets with a strong agonist combination (thrombin, epinephrin, ADP, collagen, and

TABLE II. GPIIb/IIIa Binding Sequences Selected From the CX_6C Library*

							Elution method†
Class 1	CysArg	Gly	Asp	Met	Phe	GlyCys	1
	Arg	Gly	Asp	Phe	Leu	Asn	1
	Arg	Gly	Asp	Met	Leu	Arg	1
	Arg	Gly	Asp	Ala	Phe	Gln	1
	Arg	Gly	Asp	Met	Ala	Tyr	2
Class 2	CysAsn	Trp	Lys	Arg	Gly	AspCys	2
	Asn	Thr	Leu	Lys	Gly	Asp	1
	Phe	Asn	Arg	Lys	Gly	Asp	1

*Amino acid sequences expressed on the surface of phage as GPIII fusions were deduced from DNA sequencing[37] of the region using the primer 5′ CGATCTAAAGTTTTGTCGTCT 3′.[6]
†The method used to elute bound phage in the second round of selection is noted: (1) glycine buffer (pH 2.2), or (2) 100 μM SK&F 106760.

TABLE III. Binding of Cyclic Peptides to gpIIb/IIIa

Compound	Antiaggregatory IC_{50} (μM)	Inhibition of fibrinogen binding IC_{50} (nM)
Ac-Cys-Arg-Gly-Asp-Met-Phe-Gly-Cys-$CONH_2$	3.5	700
Ac-Cys-Arg-Gly-Asp-Met-Leu-Arg-Cys-$CONH_2$	9.0	n.d.*
Ac-Cys-Arg-Gly-Asp-Phe-Leu-Asn-Cys-$CONH_2$	7.5	500
Ac-Cys-Asn-Thr-Leu-Lys-Gly-Asp-Cys-$CONH_2$	0.30	20
Ac-Cys-Asn-Trp-Lys-Arg-Gly-Asp-Cys-$CONH_2$	0.15	50
Ac-Cys-N-Methyl-Arg-Gly-Asp-Pen-$CONH_2$ (SK106760)	4.0	
Ac-Ser-Asn-Trp-Lys-Arg-Gly-Asp-Ser-$CONH_2$	140	

*n.d., not determined.

arachidonate) (Table III). The IC_{50}s of the most potent compounds were an order of magnitude lower than the fibrinogen antagonist SK&F 106760, which is a potent antithrombotic agent. The SK&F 106760 cyclic peptide, which was developed only after synthesizing an extensive array of peptides,[30] contains two unusual amino acids, penicillamine and *N*α-methyl arginine, the latter of which is difficult to synthesize and expensive to produce commercially. In contrast, the peptides discovered by the phage method are composed exclusively of common, L-amino acids and can be easily produced by chemical methods.

To verify that the oxidized form of these peptides was the active species, peptides were assayed for platelet aggregatory activity under reducing conditions. The IC_{50} values for the peptides under reducing conditions were from 2- to 20-fold higher than for the fully oxidized peptides. However, since the peptides oxidize readily under the conditions used to assay activity, a control peptide was synthesized. The control peptide substituted Ser residues for Cys to eliminate the potential for cyclization (Ser-Asn-Trp-Lys-Arg-Gly-Asp-Ser). This peptide had an IC_{50} value three orders of magnitude higher than the parent Cys peptide indicating that the oxidation of Cys residues is required for activity.

It is interesting to note that in all but one of the selected peptides the RGD or KGD sequence directly abuts one of the Cys residues. This observation is consistent with a recent computational and NMR study of a cyclic RGD peptide, which showed that the peptide in one of the low energy structures was in a somewhat extended conformation between the Arg and the Asp.[34,35] If, in their receptor bound conformations, the peptides identified here adopt an antiparallel β-hairpin with a turn centered about the two middle residues, this would position the RGD sequence in an extended conformation along either the N-terminal or C-terminal β-strand, depending on its position in the sequence. Spectroscopic studies are currently in progress to test this possibility.

CONCLUSIONS

Early studies with peptide libraries illustrated the potential of this approach for determining the specificities of antipeptide antibodies. Our results extend these early findings and, for the first time demonstrate (1) phage peptide libraries can be used to discover ligands for biologically important cellular receptors; (2) cyclic conformational constraints can be used to increase the affinity of phage-bound peptides; (3) this approach has the potential to very rapidly generate leads for therapeutically useful drugs; and (4) this approach has the potential to generate unexpected, novel leads. For instance, until recently it was thought that the RGD sequence was necessary for tight binding to IIb/IIIa. This is be-

cause, in most IIb/IIIa binding peptides, substitution of Lys for Arg results in a reduction in affinity (although homolysine or *p*-aminomethylphenylalanine can often successfully substitute for Arg).[34,35] Nevertheless, the snake venom protein, barbourin, which binds IIb/IIIa with very high affinity and impressive specificity, contains a KGD sequence.[36] The KGD peptides described in this report are the first examples of short peptides containing a Lys for Arg substitution that bind to IIb/IIIa, and illustrate the exquisite specificity of this scheme for selecting peptide sequences from a large peptide library.

ACKNOWLEDGMENTS

The authors thank M. Kendall, S. Stack, and P. Strzemienski for technical assistance. We also thank G. Koukedis and S. Kamerkar for amino acid analyses, S. Hassel, J. Bozarth, and B. Loreli for peptide activity assays, and G. Smith for helpful discussions.

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