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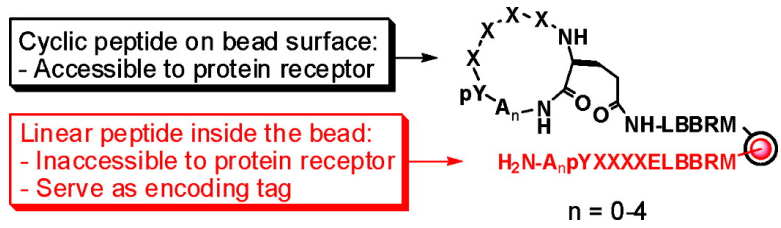

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Cyclic Peptidyl Inhibitors of Grb2 and Tensin SH2 Domains Identified from Combinatorial Libraries

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Cyclic peptides provide attractive lead compounds for drug discovery and excellent molecular probes in biomedical research. In this work, a novel method has been developed for the high-throughput synthesis, screening, and identification of cyclic peptidyl ligands against macromolecular targets. Support-bound cyclic phosphotyrosyl peptide libraries containing randomized amino acid sequences and different ring sizes (theoretical diversity of 3.2×10^6) were synthesized and screened against the SH2 domains of Grb2 and tensin. Potent, selective inhibitors were identified from the libraries and were generally more effective than the corresponding linear peptides. One of the inhibitors selected against the Grb2 SH2 domain inhibited human breast cancer cell growth and disrupted actin filaments. This method should be applicable to the development of cyclic peptidyl inhibitors against other protein domains, enzymes, and receptors.

Introduction

Cyclic peptides are a rich source of biologically active compounds and are widely produced in nature by plants, bacteria, fungi, marine invertebrates, and primate leukocytes.[1] Several cyclic peptides such as cyclosporin A (an immunosuppressant), caspofungin (an antifungal agent), and daptomycin (an antibiotic) are clinically used therapeutic agents.[2] Compared to linear peptides, cyclic peptides have reduced conformational freedom, which makes them more resistant to proteolytic degradation and potentially tighter-binding and more-specific ligands of macromolecular receptors. Given their potential as drugs, drug leads, and molecular tools in biomedical research, there has been great interest in the generation of cyclic peptides (or cyclic peptidomimetics) with improved or new biological activities by either modification of naturally occurring cyclic peptides[3] or de novo synthesis.[4] In principle, large combinatorial libraries of cyclic peptides may be readily synthesized by the split-and-pool method.[5] However, compared to the vast literature on linear peptide libraries, there have been relatively few reports on combinatorial cyclic peptide libraries (especially N-to-C cyclized peptide libraries).[6] This, we believe, is primarily because, until very recently,[7] postscreening identification of cyclic peptide hits had been technically challenging by Edman degradation (because cyclic peptides do not have a free N-terminus) or tandem mass spectrometry (because of the formation of multiple mass degenerate molecular ions upon ring opening). We recently developed a general methodology for the combinatorial synthesis, encoding, screening, and postscreening identification of cyclic peptides.[7] In this method, each resin bead (e.g., TentaGel) is spatially segregated into outer and inner layers, with a cyclic peptide displayed on the bead surface and the corresponding linear peptide restricted to the bead interior. During library screening against a macromolecular target, which is too large to diffuse into the bead, only the cyclic peptide on the bead surface is accessible to the target. After a positive bead is selected, the identity of the cyclic peptide on that bead is determined by sequencing the linear peptide in the inner core by partial Edman degradation/mass spectrometry (PED/MS).[8]

Src homology-2 (SH2) domains are small modular domains of ~100 amino acids, which mediate phosphorylation-dependent protein–protein interactions.[9] They bind to their partner proteins by recognizing specific, short phosphotyrosine (pY)-containing motifs and thereby couple protein tyrosine kinases to downstream signaling events. X-ray crystal structures show that pY peptides typically bind to SH2 domains in extended conformations, with residues from pY−2 (two residues N-terminal to pY, which is defined as position 0) to pY+5 making potential contacts with the SH2 domain surface.[10] A key interaction involves the insertion of the pY side chain into a deep pocket within the SH2 domain, which contributes the majority of the binding energy and ensures that the SH2 domain acts as a phosphorylation-dependent molecular switch. Additional binding energy and sequence specificity are provided by interactions between amino acids adjacent to pY and the less conserved regions of the SH2 domain surface. Many of the ~120 human SH2 domains have been implicated in diseases and conditions; therefore, SH2 domains provide novel targets for drug design.[11] For example, inhibitors against Src kinase SH2 domain inhibit osteoclast-mediated resorption of bone and provide a potential treatment for osteoporosis.[12]

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Grb2 is an adaptor protein composed of two SH3 domains that flank a single SH2 domain. The Grb2 protein interacts with activated growth factor receptors via its SH2 domain and with guanine nucleotide exchange factor son of sevenless (SOS) through its SH3 domain. Because of its involvement in the mitogenically important Ras signaling pathways, Grb2 provides an attractive target for the design of inhibitors as anticancer agents.[13] Tensin is a cytoplasmic protein that bridges focal adhesion to actin filaments.[14] In addition to its structural role, tensin also functions in signal transduction.[15] Tensin contains a single SH2 domain that binds to a large number of pY proteins,[16] but most of these pY proteins remain unidentified. We have previously shown that a linear pY peptide against the tensin SH2 domain disrupted the actin filaments in NIH 3T3 cells.[16d] However, the linear pY peptide was rapidly degraded during extended incubation in the cell culture (>6 h). This prompted us to develop cyclic peptides, which are metabolically more stable, as molecular probes for studying the cellular function of SH2 domains.

A large number of inhibitors have already been developed against the Grb2 SH2 domain including cyclic peptidyl inhibitors.[13] However, most of the reported cyclic inhibitors were "rationally" designed, on the basis of prior knowledge about the structures of the SH2 domain/ligand complexes. For other SH2 domains (or other macromolecular targets) whose ligands or structures are unknown, rational design is more challenging. Combinatorial chemistry offers a powerful alternative approach to inhibitor design and can be carried out in the absence of any information about the structure or ligand of a macromolecular target. In this work, we demonstrated the feasibility of the library approach by synthesizing and screening two cyclic peptidyl libraries against the SH2 domains of Grb2 and tensin to identify potent, biologically active cyclic peptidyl inhibitors against these two domains.

Results

Design and Synthesis of Cyclic Peptide Libraries. The sequence specificities of Grb2 and tensin SH2 domains have previously been determined by screening linear peptide libraries. Grb2 SH2 domain requires an Asn at the pY+2 position but tolerates a variety of amino acids at other positions.[17] Tensin SH2 domain binds to three distinct classes of pY peptides of the consensuses pY(D/E)N(V/M/Y/L) (class I), pY(Y/F/L)φ(Y/F/M) (class II), and pYY(E/D)N (class III) (where φ represents hydrophobic amino acids).[16b,d] Initially, we did not know whether the size of the ring would affect binding to the SH2 domains or what the optimal ring size might be. Therefore, we arbitrarily chose a cyclic pY peptide library containing 10 amino acids, cyclo(AXX-pYXXXLNE)BBRM-resin [library I; where B is β-alanine and X is 2-L-aminobutyrate (Abu, as a cysteine surrogate), norleucine (Nle, as a methionine replacement), or any of the 18 proteinogenic amino acids except for Cys and Met]. Later, to determine the optimal ring size for the SH2 domains, we constructed another library, cyclo(A_npYXXXXE)LBBRM (library II), in which different number of alanines ($n = 0-4$) were added to the N-terminal side of pY to generate cyclic peptides of 6−10 amino acids. The linker sequence, LBBRM, was included to facilitate peptide release (after Met by CNBr) and MALDI MS analysis (Arg provides a positive charge, and the added mass shifts peptide signals to m/z > 500 region). The invariant Glu residue was attached to the linker sequence via its side chain, leaving its α-carboxyl group available for later N-to-C peptide cyclization (Figure 1). The theoretical diversity of libraries I and II was 20^5 or 3.2×10^6 and 5×20^4 or 8.0×10^5, respectively.

The libraries were synthesized on TentaGel S NH_2 resin (90 μm, $\sim 2.86 \times 10^6$ beads/g, ~100 pmol/bead) (Figure 1). To facilitate postscreening hit identification, each TentaGel bead was spatially segregated into outer and inner layers, with cyclic peptides displayed on the bead surface whereas the corresponding linear peptides confined in the inner core. This was accomplished by quickly suspending the TentaGel resin that had been equilibrated in an aqueous solution in 55:45 (v/v) dichloromethane/diethyl ether containing 0.5 equivalent of N^{α}-Fmoc-Glu(δ-NHS)-O-$CH_2CH{=}CH_2$) (Figure 1). Because the organic solvents were immiscible with water, the beads became phase separated; their surface layer was quickly re-equilibrated in the organic solvents, while the inner core remained in the aqueous environment. Thus, only peptides on the bead surface were exposed to and reacted with the activated ester. The remaining free N-terminal amine in the bead interior (0.5 equivalent) was subsequently acylated with Fmoc-Glu(tBu)-OH. The random sequence was synthesized by the split-and-pool method to give one-bead one-compound (OBOC) libraries. After the addition of pY and varying number of alanines, the N-terminal Fmoc group and the C-terminal α-allyl group (on the C-terminal Glu) were removed and the surface peptides were cyclized by treatment with benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), while the inner peptides were kept in the linear form.

Cyclic Peptide Ligands of Grb2 SH2 domain. Library screening was based on an on-bead enzyme-linked assay,[17e] in which binding of the biotinylated SH2 domain to a bead that contains a high-affinity ligand recruits a streptavidin-alkaline phosphatase conjugate to the bead. Upon the subsequent addition of 5-bromo-4-chloro-3-indolyl phosphate (BCIP), the phosphatase hydrolyzes BCIP to generate a turquoise precipitate deposited on the bead surface. A total of 90 mg of library I ($\sim 2.5 \times 10^5$ beads) was screened against the Grb2 SH2 domain and 58 most colored beads were removed from the library. To ensure that the positive beads were selected as a result of binding of the SH2 domain to cyclic peptides (instead of any uncyclized peptides) on the bead surface, the 58 beads were treated exhaustively with a nonspecific aminopeptidase, *Aeromonas* aminopeptidase (AAP), which should remove any surface exposed linear peptides.[18] The AAP-treated beads were subjected to a second round of screening against the Grb2 SH2 domain and the resulting positive beads (55 beads) were sequenced by PED/MS (see Figure S1 in Supporting Information for representative MS spectra) to give 25 complete sequences (Table 1). Most of the selected sequences contained an Asn at the pY+2 position, in agreement with earlier studies.[17] At the pY+3 position, Grb2 SH2 domain strongly prefers a hydrophobic residue (e.g., Val, Tyr, Ile), although Gln was

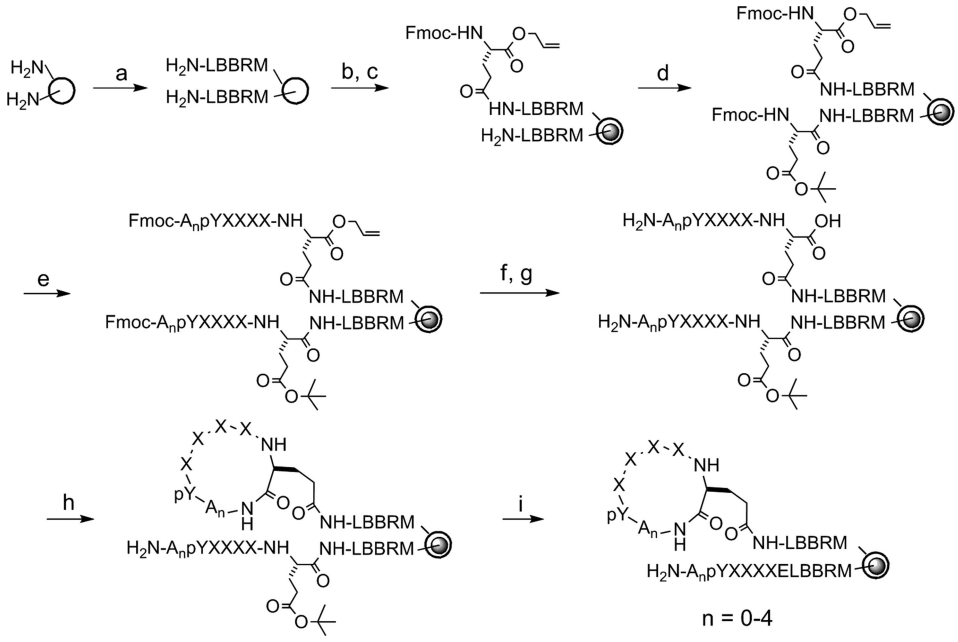

Figure 1. Synthesis of cyclic peptide library with different ring sizes: (a) standard Fmoc/HBTU chemistry, (b) soak in water; (c) 0.5 equiv N^{α}-Fmoc-Glu(δ-NHS)-O-$CH_2CH{=}CH_2$ in Et_2O/CH_2Cl_2, (d) excess Fmoc-Glu(OtBu)-OH, HBTU, (e) split-and-pool synthesis by Fmoc/HBTU chemistry, (f) $Pd(PPh_3)_4$, (g) piperidine, (h) PyBOP, HOBt, and (i) TFA.

Table 1. Binding Sequences Selected from Cyclic pY Library I and II against Grb2 and Tensin SH2 Domains[a]

Grb2 SH2 domain			tensin SH2 domain		
library I	library II		library I	library II	
RTpYKNF	6-mer	9-mer	RRpYCNV	7-mer	AApYENFQ
RRpYCNI	pYCHQH	AAApYANIR	RHpYDNC	ApYFFIH	AApYENVF
RRpYENI	pYQNIR	AAApYCARA	SFpYDNF	ApYYFQM	AApYMNYY
RKpYQNI	pYQNFK	AAApYHGTF	LGpYDNI	ApYANVY	AApYMNFP
GYpYVNI	pYVNCR	AAApYINYH[b]	IMpYDNL	ApYDPSR	AApYVNVS
YKpYRNL		AAApYKNMI	HHpYDNM	ApYVTAP	AApYVNYA
HFpYANV	7-mer	AAApYKNVH	FWpYDNT	ApYHYVN	AApYYNFV[b]
FNpYCNV	ApYRNIG	AAApYQNYF[b]	FFpYDNV		AApYRRFR
FCpYCNV[b]	ApYSNYK	AAApYVNMR	HHpYDNV	8-mer	AApYTRKR
PRpYKNV		AAApYYNLR	NIpYDNV[b]	AApYWAVH	AApYQYIQ
HCpYMNV	8-mer		THpYDNV	AApYYEHF	AApYYYLM
RRpYRNV	AApYKNYP	10-mer	YFpYENC	AApYSINI	
VIpYANY	AApYKNYR	AAAApYANVV	HDpYENV	AApYEMVH	9-mer
YApYCNY	AApYNNQF	AAAApYASPA	GGpYVNC	AApYANMR	AAApYNFSH
HGpYQNY	AApYRNFF	AAAApYINMH	MYpYSRC	AApYANFV[b]	AAApYEHVQ
IKpYQNY[b]	AApYRNFM	AAAApYMNIR	CGpYYCN	AApYCNCH	AAApYDNYM
KFpYYNY	AApYVNFF[b]	AAAApYNNYF	FSpYYEN	AApYDNLR	AAApYENVH
HRpYCNT		AAAApYQNCM	FNpYYYN	AApYDNCH	AAApYRNDI
HWpYMNT		AAAApYQNYA		AApYDNVH	AAApYTTYQ
HTpYCNQ		AAAApYRNIC		AApYDNYM[b]	AAApYDYAR
HTpYCNQ		AAAApYRNLI		AApYDNMQ	AAApYYYNV
AYpYVNQ		AAAApYVNLA		AApYENSY	
FRpYACQ		AAAApYVNMP		AApYENHC	10-mer
GGpYMAQ				AApYENHP	AAAApYDNFC
LNpYQRM				AApYENCA	AAAApYEYLV
				AApYENVR	

[a] C, 2-L-aminobutyrate; M, L-norleucine. [b] Peptides selected for resynthesis and binding studies to SH2 domains by SPR.

also selected in a few sequences. The Grb2 SH2 domain has no obvious selectivity at the −2, −1, and +1 positions.

Cyclic Peptide Ligands of Tensin SH2 domain. Library I (90 mg) was similarly screened against the tensin SH2 domain at three different concentrations (5, 10, and 50 nM SH2 domain). Similar sequences (18 total) were selected from all three experiments (Table 1), demonstrating the reproducibility of the screening procedure. The majority of the selected sequences belong to the class I consensus pY(D/E)N(V/C).[16b,d] Like the Grb2 SH2 domain, tensin SH2 domain strongly prefers an asparagine at the pY+2 position but has little selectivity at the N-terminal side of pY. In addition, it has a strong preference for a hydrophobic residue (especially Val and Abu) at pY+3 position. However, the two domains have dramatically different specificities at the pY+1 position; while the Grb2 SH2 domain accepts a wide variety of amino acids, the tensin SH2 domain strongly prefers an acidic residue (Asp or Glu). Three of the selected peptides (pYYCN, pYYEN, and pYYYN) fall into a different class

Table 2. Dissociation Constants (K_D, nM) of Selected Peptides against Grb2 and tensin SH2 domains

no.	peptide sequence[a]	Grb2 SH2	tensin SH2
1	cyclo(AFCpYCNVLNE)-$(PEG)_2$-K(biotin)-NH_2	88 ± 12	ND[b]
2	cyclo(AIKpYQNYLNE)-$(PEG)_2$-K(biotin)-NH_2	170 ± 23	ND
3	cyclo(ANIpYDNVLNE)-$(PEG)_2$-K(biotin)-NH_2	940 ± 100	130 ± 13
4	AFCpYCNVLNE-$(PEG)_2$-K(biotin)-NH_2 (linear)	330 ± 60	ND
5	AIKpYQNYLNE-$(PEG)_2$-K(biotin)-NH_2 (linear)	880 ± 110	>10000
6	ANIpYDNVLNE-$(PEG)_2$-K(biotin)-NH_2 (linear)	1600 ± 60	230 ± 20
7	cyclo(pYQNYFE)-$(PEG)_2$-K(biotin)-NH_2	150 ± 20	ND
8	cyclo(ApYQNYFE)-$(PEG)_2$-K(biotin)-NH_2	54 ± 8	ND
9	cyclo(AApYQNYFE)-$(PEG)_2$-K(biotin)-NH_2	82 ± 12	ND
10	cyclo(AAApYQNYFE)-$(PEG)_2$-K(biotin)-NH_2	78 ± 5	ND
11	cyclo(AAAApYQNYFE)-$(PEG)_2$-K(biotin)-NH_2	700 ± 110	ND
12	cyclo(AAApYINYHE)-$(PEG)_2$-K(biotin)-NH_2	100 ± 12	ND
13	cyclo(AApYVNFFE)-$(PEG)_2$-K(biotin)-NH_2	45 ± 3	ND
14	AAApYQNYFE-$(PEG)_2$-K(biotin)-NH_2 (linear)	160 ± 20	ND
15	AAApYINYHE-$(PEG)_2$-K(biotin)-NH_2 (linear)	350 ± 40	ND
16	AApYVNFFE-$(PEG)_2$-K(biotin)-NH_2 (linear)	140 ± 20	ND
17	cyclo(AApYDNYME)-$(PEG)_2$-K(biotin)-NH_2	ND	130 ± 40
18	cyclo(AApYYNFVE)-$(PEG)_2$-K(biotin)-NH_2	ND	140 ± 20
19	cyclo(AApYANFVE)-$(PEG)_2$-K(biotin)-NH_2	ND	1700 ± 100
20	AApYDNYME-$(PEG)_2$-K(biotin)-NH_2 (linear)	ND	350 ± 45
21	AApYYNFVE-$(PEG)_2$-K(biotin)-NH_2 (linear)	ND	850 ± 90
22	AApYANFVE-$(PEG)_2$-K(biotin)-NH_2 (linear)	ND	660 ± 120

[a] C, 2-L-aminobutyrate; M, L-norleucine. [b] ND, not determined.

(class II). These results are very similar to those previously obtained from a linear peptide library.[16d]

Binding Affinities of the Selected Cyclic Peptide Ligands. Two of the cyclic peptides selected against the Grb2 SH2 domain [cyclo(AFCpYCNVLNE) and cyclo(AIKpYQNYLNE) (Table 2, peptides 1 and 2)], one of the tensin SH2 domain-binding peptides [cyclo(ANIpYDNVLNE) (peptide 3)], and their corresponding linear peptides (peptides 4−6) were individually synthesized and tested for binding to the SH2 domains by surface plasmon resonance (SPR). The peptides were derivatized at the side chain of the invariant Glu residue with a flexible bis(8-amino-3,6-dioxaoctanoyl) [$(PEG)_2$] linker, followed by a lysine residue. A biotin was added to the lysyl side chain and the resulting peptides were immobilized to streptavidin-coated SPR sensorchips. Increasing concentrations of the SH2 domain proteins were passed over the sensorchips and the dissociation constant (K_D) was determined from the relationship between equilibrium response units (RU) and the protein concentration. All three cyclic peptides bound to their cognate SH2 domains with high affinity (K_D = 88−170 nM) (Table 2). For the Grb2 SH2 domain, the two selected cyclic peptide ligands bound the domain with approximately 4-fold higher affinity than their linear counterparts (Table 2, compare peptides 1 and 4, 2, and 5). Tensin SH2 domain bound to its cyclic ligand with slightly higher affinity than to the linear control (1.8-fold). We also noted that the cyclic peptides bound to tensin SH2 domain with slower kinetics (lower k_{on} and k_{off} rates) than the corresponding linear peptides (Figure S2 in Supporting Information). Consistent with the observed broader specificity of Grb2 SH2 domain relative to the tensin SH2 domain, Grb2 SH2 domain bound to peptide 3, which was selected against the tensin domain, with a respectable affinity (K_D = 940 nM), whereas the tensin SH2 domain bound to the Grb2 ligands (peptides 2 and 5) only weakly (K_D > 10000 nM).

Effect of Ring Size on the Sequence and Affinity of Cyclic Peptide Ligands. To assess the effect of peptide ring size on binding affinity, we screened library II against Grb2 and tensin SH2 domains. Again, positive beads from the primary screening were treated with AAP and the screening was repeated in the presence of 4 μM competing peptide ARVpYENVGL, which binds to both Grb2 and tensin SH2 domains. The addition of a competing peptide has been shown to increase the screening stringency and facilitate the identification of the tightest binding ligands.[19] By using this two-stage screening procedure, we screened 100 mg of library II against each domain and obtained 32 and 44 binding sequences for Grb2 and tensin SH2 domain, respectively (Table 1). Again, the Grb2 SH2 domain showed overwhelming preference for an Asn at the pY+2 position and hydrophobic residues at the pY+3 position (Figure 2). The tensin SH2 domain prefers an Asn residue at the pY+2 position and an acidic residue (Asp or Glu) at the pY+1 position. Both domains favor hydrophobic and aromatic residues (e.g., Val, Phe, or Tyr) at the pY+3 position. The tensin SH2 domain also has some preference for hydrophilic residues such as His, Arg, and Gln at the pY+4 position. Interestingly, the two domains exhibited different ring-size preferences. The Grb2 SH2 domain can tolerate a wide range of ring sizes (6−10 amino acids), although it appears to prefer larger ring sizes (8−10 amino acids). Among the selected peptides, 34%, 28%, and 18% were deca-, nona-, and octapeptides, respectively (Table 1). Only six of the selected peptides (20%) were hepta- or hexapeptides. The tensin SH2 domain has a narrower ring size range and strongly prefers octapeptides, which made up 64% of the selected peptides. Nona- and heptapeptides came next at 18% and 14%, respectively. No hexapeptide and only two decapeptides were selected from the library, although peptides with the five different ring sizes were equally represented in library II (20% each). There was also a correlation between selected peptide sequences and the ring size. For example, while the cyclooctapeptides selected against the tensin SH2 domain were dominated by sequences of the consensus pY(D/E)Nφ (where φ represents hydrophobic amino acids), none of the heptapeptides contained this motif (Table 1).

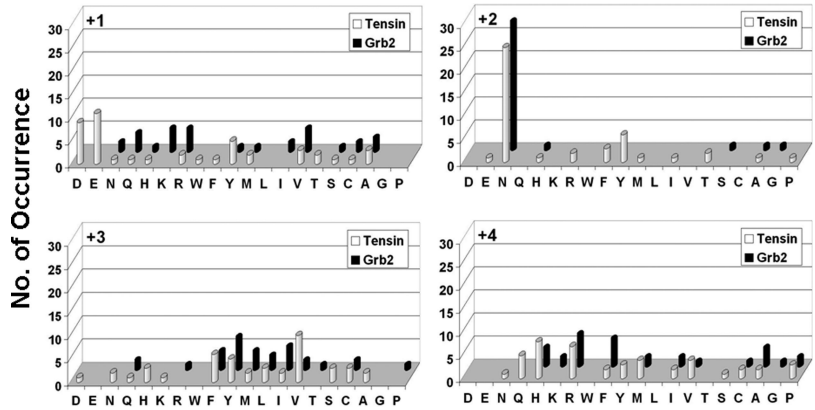

Figure 2. Sequence specificities of tensin and Grb2 SH2 domains. The histograms represent the amino acids identified at each position from pY+1 to pY+4. Number of occurrence on the *y* axis represents the number of selected sequences that contain a particular amino acid at a certain position. Key: white bar, tensin SH2 domain; black bar, Grb2 SH2 domain; M, Nle; C, Abu.

One of the octapeptides [cyclo(AApYVNFFE) (Table 2, peptide 13)] and two of the nonapeptides selected against the Grb2 SH2 domain [cyclo(AAApYQNYFE) (peptide 10) and cyclo(AAApYINYHE) (peptides 12)] and their linear counterparts (Table 2, peptides 14−16) were resynthesized and their binding affinities to the Grb2 SH2 domain were determined by SPR. As expected, all three cyclic peptides were potent ligands of Grb2 SH2 domain (K_D = 45−100 nM) and bound 2−4-fold more tightly than the linear counterparts. Next, peptide 10 was chosen for further studies to test the ring size preference of the Grb2 SH2 domain. Five cyclic peptides that contain the same pYQNYFE motif but different numbers of alanines to give ring sizes of 6−10 amino acids were synthesized (Table 2, peptides 7−11). Among the five peptides, the hepta-, octa-, and nonapeptides had the highest binding affinities, with K_D values of 54−82 nM, whereas the hexa- and decapeptides were less potent (K_D = 150 and 700 nM, respectively). Three octapeptides selected against the tensin SH2 domain were also resynthesized and analyzed. Two of the peptides, cyclo(AApYDNYME) (Table 2, peptide 17) and cyclo(AApYYNFVE) (peptide 18), bound to tensin SH2 domain more tightly than their linear counterparts (by 3−6-fold), whereas the third peptide, cyclo(AApYANFVE) (Table 2, peptide 19), was ∼2-fold less potent than the corresponding linear sequence (Table 2 compare peptides 19 and 22). Thus, our results demonstrate that at least for the two SH2 domains tested in this work, it is possible to generate highly potent and specific cyclic peptide ligands by the combinatorial approach. Our data also indicate that there is no universally optimal ring size because different SH2 domains prefer different ring sizes. Even for a single SH2 domain, the most optimal ring depends on the actual peptide sequence. This complex relationship between the peptide sequence and ring size highlights the importance of being able to simultaneously vary the ring size and amino acid sequence in a combinatorial library.

Inhibition of Cellular Events by Selected Cyclic Grb2 SH2 Ligands. Octapeptide cyclo(AApYVNFFE) (Table 2, peptide 13), which had the highest affinity for the Grb2 SH2 domain among the tested peptides, was selected for cellular studies. Grb2 is a key player in many growth factor receptor-mediated signaling pathways.[20] For example, when cells are

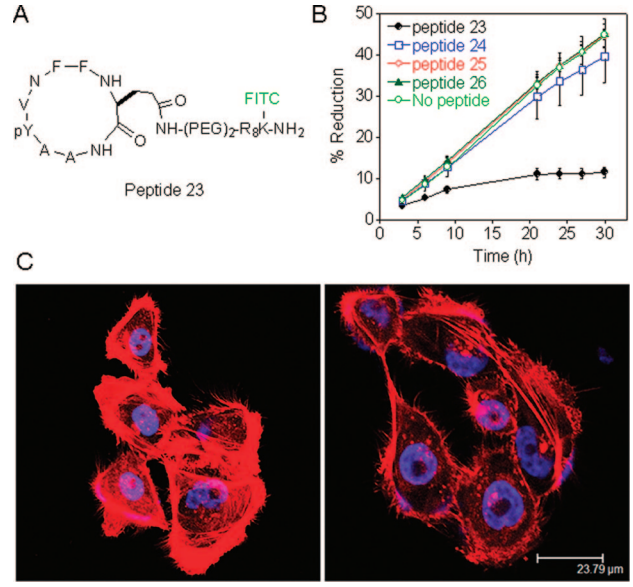

Figure 3. Cellular activities of Grb2 SH2 domain inhibitor. (A) Structure of peptide 23. Peptide 24 has the structure of Ac-AApYVNFFE-$(PEG)_2$-R_8K(FITC)-NH_2. (B) Effect of peptide treatment on the growth rate of SUM-102PT (human breast cancer) cells as monitored by the amount of AlamarBlue reduced. Experiments were performed in triplicates and data presented were mean ± standard deviation. (C) Effect of peptide treatment on the cytoskeleton of SUM-102PT cells. Left panel, no peptide treatment; right panel, cells treated with 20 μM peptide 23 for 3 h. Actin filaments were stained by rhodamine-phalloidin (red), whereas the nuclei were stained with DAPI (blue).

stimulated with epidermal growth factor (EGF), Grb2 SH2 domain binds (or through Shc) to tyrosine phosphorylated EGF receptor (EGFR) and activates the Ras signaling pathway, resulting in cell proliferation and assembly of actin filaments.[21] An inhibitor against the Grb2 SH2 domain should terminate the EGFR signaling pathway, disrupt the actin cytoskeleton, and halt cell proliferation.[22] To make the octapeptide permeable to the cell membrane, an octaarginine sequence (R_8K-NH_2) was appended to the side chain of the Glu via a flexible $(PEG)_2$ linker (Figure 3A, peptide 23). To monitor peptide uptake by cells, fluorescein isothiocyanate (FITC) was conjugated to the side chain of the C-terminal lysine. The corresponding linear peptide, Ac-AApYVNFFE-$(PEG)_2$-R_8K(FITC)-NH_2 (peptide 24), was also synthesized for comparison. In addition, two control

peptides (peptides 25 and 26) were prepared. Peptide 25 was identical to the peptide 23, except that it contained a Tyr in replacement of the pY residue. Peptide 26 contained a Gln instead of the critical Asn at the pY+2 position and was otherwise identical to peptide 23. Both peptides 25 and 26 should have greatly reduced affinity to the Grb2 SH2 domain and thus have no or less inhibitory effects in cellular assays.

Human breast cancer cells were treated with the above peptides and cell proliferation was monitored by AlamarBlue assay, which measures the reduction of a blue nonfluorescent dye resazurin in cell culture resulting in absorbance at 570 nm.[23] Treatment of the cells with peptide 23 resulted in dose-dependent reduction in cell growth rate, with 80% growth inhibition at 20 μM peptide (Figure 3B and Figure S3 in Supporting Information). In comparison, the linear pY peptide (peptide 24), which has only 3-fold lower affinity than peptide 23 (Table 2, compare peptides 13 and 16), caused only 20% inhibition of cell growth under the same conditions. Control peptides 25 and 26 had no effect. The large difference in cellular activities between peptides 23 and 24 is likely caused by increased stability of the cyclic peptide in addition to its higher potency.

Next, peptides 23−26 were tested for their effect on the actin skeletal structure in the breast cancer cells. Treatment of the cells with 20 μM cell-permeable peptides (23−26) resulted in intense green fluorescence inside the cells, indicating that the peptides were efficiently taken up by the cells (data not shown). The cells were then washed and stained with rhodamine-phalloidin and counterstained with DAPI to visualize the actin filaments and the nuclei, respectively. Without peptide treatment, actin filaments were readily visible in >80% of the cells (Figure 3C). However, among cells that were treated with 20 μM cyclic peptide 23 for 3 h, only ~40% displayed visible actin filaments. The retention of actin skeletal structures in this cell population may be the result of poorer peptide uptake by these cells or increased peptide degradation (e.g., by phosphatases and proteases). Treatment with 20 μM linear peptide 24 also resulted in loss of actin filaments but to a lesser extent than that for peptide 23 (Figure S4 in Supporting Information). Cells treated with peptides 25 and 26 displayed similar cytoskeleton features as the untreated cells. Taken together, our data suggest that cyclic peptide 23 is a specific inhibitor of the Grb2 SH2 domain and that the observed cell growth inhibition and disruption of actin filaments are direct results of inhibiting the Grb2 SH2 domain by this peptide.

Discussion

In this work, we employed a combinatorial library approach to develop cyclic peptide inhibitors against two SH2 domains from Grb2 and tensin. Although cyclic peptide ligands have previously been developed for the Grb2 SH2 domain,[13] no such ligands have been reported for tensin SH2 domain. As expected, all of the selected cyclic peptides bound to their cognate SH2 domains (i.e., the domain against which they were selected) with high affinity. Furthermore, despite that the two SH2 domains have very similar sequence specificities, some of the cyclic peptides showed respectable selectivity for their cognate SH2 domain. We showed that cyclic peptide 23 (which was selected against the Grb2 SH2 domain) is active in cellular assays, capable of inhibiting actin polymerization and tumor cell growth. These outcomes are consistent with the specific inhibition of the Grb2 SH2 domain by the cyclic peptide. These inhibitors will provide invaluable tools for elucidating the biological function of Grb2 and tensin in cellular processes. Compared to biological methods commonly used for cellular perturbation (e.g., gene knockout and RNAi), which eliminate the entire protein of interest from a system, a chemical probe permits one to inhibit a specific function of the protein and examine its biological effects. Chemical probes also have potentially much better temporal resolution. Of course, the inhibitors may be used in combination with the biological methods. In addition, the cyclic peptide ligands may provide potential therapeutic agents (e.g., Grb2 inhibitors as anticancer drugs).

Cyclic peptides have been prepared through a variety of methods. First, biologically active cyclic peptides have been generated by de novo design[4] or by modification of natural products.[3] In this method, peptides are usually synthesized individually (by sequential or parallel synthesis) and the number of peptides that can be practically synthesized is limited. Second, cyclic peptide libraries have been biologically synthesized by in vivo (e.g., phage display[24] and intein-based peptide cyclization)[6d,25] or in vitro translation (e.g., mRNA display).[26] In phage display, peptides are typically fused to the N-terminus of a phage coat protein, and cyclization is mediated by disulfide formation between a pair of cysteines that flank the random peptide sequence. During mRNA display, each peptide is first synthesized in the linear form by in vitro translation and subsequently cyclized by disulfide formation or treatment with a selective cross-linking agent (e.g., an N-hydroxysuccinimide ester).[26] Intein-based cyclization takes advantage of the ability of intein proteins to splice peptide segments; for peptide cyclization, the two exteins are covalently linked.[6d,24b] While powerful, these biological methods are generally limited to the 20 proteinogenic amino acids (although several methods have recently been developed to incorporate unnatural building blocks into biologically synthesized peptide libraries).[27] In the case of phage display, cyclization is also limited to disulfide formation, which is not stable inside cells. Third, OBOC peptide libraries have been chemically synthesized and cyclized through amino acid side chains (via disulfide or other cross-linkers).[28] The resulting cyclic peptides retain free N-termini and therefore can be sequenced by Edman degradation. Compared to the earlier methods, our library method is more general and does not suffer from the above limitations. For example, our method can readily accommodate unnatural amino acids and peptidomimetics because our libraries are chemically synthesized. This feature will be especially important for generating biologically active and metabolically stable compounds. Although we have so far only employed N-to-C cyclization in our work, our method is readily compatible with any of the end-to-side chain or side chain-to-side chain cyclization strategies. Our method is also compatible with solution-phase screening.[3d,7]

In conclusion, we have developed a high-throughput methodology for the synthesis and screening of cyclic

peptides against macromolecular targets for desired biological activities. In the current work, we applied this new methodology to develop potent, selective cyclic peptidyl inhibitors against the SH2 domains of Grb2 and tensin. One of the selected inhibitors against the Grb2 SH2 domain was shown to disrupt the actin cytoskeleton and inhibit the growth of human breast cancer cells. To our knowledge, this is the first example of a cyclic peptide library in which both the peptide sequence and the ring size are simultaneously randomized. Our results demonstrate that both the peptide sequence and the size of the macrocycle are critical for tight binding to a macromolecular target. This method should be generally applicable to other protein modular domains, enzymes, protein receptors, and other macromolecules (e.g., RNA).

Experimental Section

Materials. The pMAL-c2 vector, all DNA modifying enzymes, and amylose resin were purchased from New England Biolabs (Ipswich, MA). All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). BCIP, antibiotics, *N*-hydroxysuccinimido-biotin, Sephadex G-25 resin, 4-hydroxy-α-cyanocinnamic acid and organic solvents were obtained from Sigma-Aldrich (St. Louis, MO). Talon resin for IMAC purification was purchased from Clontech (Mountain View, CA). Reagents for peptide synthesis were from Advanced ChemTech (Louisville, KY), Peptides International (Louisville, KY), and NovaBiochem (La Jolla, CA). Protein concentration was determined by the Bradford method using bovine serum albumin as standard. Human breast cancer cell line SUM-102PT cells were purchased from Asterand (Detroit, MI) and maintained in Ham's F-12 medium. AlamarBlue was purchased from Biotium Inc. (Hayward, CA). Rhodamine-phalloidin was purchased from Cytoskeleton (Denver, CO), and DAPI was from Vector laboratories (Burlingame, CA).

Expression, Purification, and Biotinylation of SH2 Domains. The human tensin SH2 domain was expressed as a fusion protein with the maltose-binding protein (MBP) or with an N-terminal six-histidine tag as previously described.[16d] The DNA fragment coding for the Grb2 SH2 domain (amino acids 58–151) was isolated by polymerase chain reaction (PCR) from the human fetus Marathon-Ready cDNA library (Clontech) with the following primers: 5′-GGCTATCGAATTCCATCCGTGGTTTTTTG-GCAAAATCCCC-3′ and 5′-GGGTTAAGCTTTTATAT-GTCCCGCAGGAATATCTGCTGGTTTC-3′. The PCR product was digested with restriction endonucleases *Eco*RI and *Hind*III, and ligated into the corresponding sites in vector pMAL-c2. This procedure resulted in the fusion of the SH2 domain to the C-terminus MBP, facilitating both purification and biotinylation. The identity of the DNA constructs was confirmed by dideoxy sequencing. Expression, purification, and biotinylation of SH2 domains were performed as previously described.[29]

Library Synthesis. Library II was synthesized on 2.0 g of TentaGel S NH_2 resin (90 μm, 0.26 mmol/g). All of the manipulations were performed at room temperature unless otherwise noted. The linker sequence (LBBRM) was synthesized with 4 equiv of Fmoc-amino acids, using HBTU/HOBt/*N*-methylmorpholine (NMM) as the coupling reagents. The coupling reaction was typically allowed to proceed for 1.5 h, and the beads were washed with DMF (3×) and DCM (3×). The Fmoc group was removed by treatment twice with 20% piperidine in DMF (5 + 15 min), and the beads were exhaustively washed with DMF (6×). To segregate the beads into outer and inner layers, the beads were soaked in water overnight, drained, and suspended in 30 mL of 55:45 (v/v) DCM/diethyl ether containing N^{α}-Fmoc-Glu(δ-N-hydroxysuccinimidyl)-O-$CH_2CH{=}CH_2$ (0.3 mmol, 0.50 equiv)[7] and diisopropylethylamine (0.3 mmol). The mixture was incubated on a rotary shaker for 30 min. After it was washed with 55:45 DCM/diethyl ether (3×) and DMF (8×), the resin was treated with 2 equiv of Fmoc-Glu(tBu)-OH plus HBTU/HOBt/NMM in DMF (90 min). For the synthesis of random residues, the resin was split into 20 equal portions, and each portion (100 mg) was coupled twice, each with 5 equiv of a different Fmoc-amino acid/HBTU/HOBt/NMM for 1 h. To differentiate isobaric amino acids during MS sequencing, 5% (mol/mol) CD_3CO_2D was added to the coupling reactions of Leu and Lys, whereas 5% $CH_3CD_2CO_2D$ was added to norleucine reaction.[8] After the addition of pY residue, the resin was divided into five equal portions. A different number of alanines (0–4) were added to each portion to produce a library with different ring sizes. The allyl group on the C-terminal glutamate was removed by overnight treatment with a solution containing tetrakis(triphenylphosphine)palladium (1 equiv), triphenylphosphine (3 equiv), formic acid (10 equiv), and diethylamine (10 equiv) in anhydrous THF. The beads were washed with 0.5% diisopropylethylamine in DMF, 0.5% sodium dimethyldithiocarbamate hydrate in DMF, DMF (3×), DCM (3×), and DMF (3×). The N-terminal Fmoc group was then removed with 20% piperidine, and the beads were washed with DMF (6×), 1 M HOBt in DMF (3×), DMF (3×), and DCM (3×). For peptide cyclization, the resin was incubated in a solution containing PyBOP/HOBt/NMM (5, 5, and 10 equiv) in DMF for 3 h on a rotary shaker. The resin was washed with DMF (3×) and DCM (3×) and dried under vacuum for >1 h. Side-chain deprotection was carried out with a modified reagent K (6.5% phenol, 5% water, 5% thioanisole, 2.5% ethanedithiol, 1% anisole, and 1% triisopropylsilane in TFA) for 2 h. The resin was washed with TFA and DCM and dried under vacuum before storage at −20 °C. Library I was similarly synthesized.

Library Screening. A typical screening experiment involved 30–50 mg of library I or II in a micro-BioSpin column (0.8 mL, BioRad) containing 50 nM SH2 protein as previously described.[17e] The positive beads (typically ∼30 beads) were pooled in a microcentrifuge tube and washed with 2 × 1 mL of SAAP staining buffer (30 mM Tris, pH 8.5, 100 mM NaCl, 5 mM $MgCl_2$, 20 μM $ZnCl_2$) and DMF (2 × 1 mL). The beads were suspended in 1 mL of Hepes buffer (20 mM Hepes, pH 7.4, 150 mM NaCl) containing 3 units of AAP and incubated for 3 h. The beads were extensively washed with the Hepes buffer, DMF, and blocking buffer (30 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% gelatin, and 0.01% Tween 20) and subjected to a second round of screening against the SH2 domain, which

was carried out under the same conditions as the first round except that a competing peptide (ARVpYENVGL, 4 μM) was added to the binding reaction along with the SH2 domain. Bead washing was carried out by gently centrifuging the tube and decanting the supernatant carefully under a microscope to ensure that no bead was removed during washing. Positive beads from the second round of screening were sequenced by PED/MS as previously described.[8] Control experiments with biotinylated MBP produced no colored beads under identical conditions.

Synthesis of Individual pY Peptides. Each peptide was synthesized on 80 mg of Rink Resin LS (0.2 mmol/g) in a manner similar to that employed for the library construction except that spatial segregation was not necessary. After the addition of the last amino acid, the resin was split into two equal aliquots. One aliquot was used for cyclization, whereas the other was used to synthesize the corresponding linear peptide. For the preparation of cyclic peptides, the allyl group on Glu was first removed followed by the removal of the N-terminal Fmoc group. The reaction condition for peptide cyclization was identical to that used during library synthesis and the progress of cyclization was monitored by ninhydrin tests. Biotinylation of the peptides was achieved by employing commercially available Fmoc-Lys(biotin)-OH during solid-phase peptide synthesis. After cleavage and deprotection with the modified reagent K, the crude peptides were purified by reversed-phase HPLC on a C_{18} column (Vydac 300 Å, 4.6 × 250 mm) and their identity was confirmed by MALDI-TOF mass spectrometric analyses.

Determination of Dissociation Constants by SPR. All measurements were made at room temperature on a BIAcore 3000 instrument following a previously described procedure.[28] Increasing concentrations of an SH2 protein (0–5 μM) in HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% polysorbate 20) were passed over the sensorchip for 120 s (for linear peptides) or 200 s (for cyclic peptides) at a flow rate of 15 μL/min. A blank flow cell (no immobilized pY peptide) was used as control to correct for any signal from the solvent bulk or nonspecific binding interactions (no significant bulk effect or nonspecific binding was observed). In between two runs, the sensorchip surface was regenerated by flowing a strip solution (10 mM NaCl, 2 mM NaOH, and 0.025% SDS in H_2O) for 5–10 s at a flow rate of 100 μL/min. The equilibrium response unit (RU_{eq}) at a given SH2 protein concentration was obtained by subtraction of the response of the blank flow cell from that of the sample flow cell. The dissociation constant (K_D) was obtained by nonlinear regression fitting of the data to the equation

$$RU_{eq} = RU_{max}[SH2]/(K_D + [SH2])$$

where RU_{eq} is the measured response unit at a certain SH2 protein concentration and RU_{max} is the maximum response unit.

Cell Culture. Human breast cancer cell line SUM-102PT cells were maintained in Ham's F-12 medium supplemented with 5 μg/mL insulin, 10 ng/mL EGF, 1 mg/mL hydrocortisone, 5 mM ethanolamine, 10 mM HEPES, 5 μg/mL transferring, 10 nM triidothyronine, 50 nM sodium selenite, 0.5 g/L bovine serum albumin, 50 units/mL penicillin, 50 μg/mL streptomycin, and 500 ng/mL fungizone. Cells were cultured in a humidified incubator at 37 °C with 5% CO_2.

AlamarBlue Assay. SUM-102PT cells were plated in 96-well plates at 5000 cells per well in 100 μL of medium as described above and cultured at 37 °C with 5% CO_2 for 16 h. The spent medium was then replaced with fresh medium containing the individual peptide at concentrations of 0, 5, 10, or 20 μM. Each treatment was performed in triplicate. Ten microliters (10% v/v) of AlamarBlue solution was added into the medium, and they continued to incubate at 37 °C. Reduction of AlamarBlue was monitored by measuring absorptions at 570 and 600 nm using a microtiter plate reader (Molecular devices, Sunnyvale, CA) at indicated time points. Percent reduction of AlamarBlue was determined as previously described.[23]

Confocal Microscopy Imaging. SUM-102PT cells were cultured on glass coverslips in 6-well plates at 37 °C with 5% CO_2 for 16 h. After they were treated with 20 μM individual peptide for 3 h, cells were washed with phosphate buffered saline (PBS, 140 mM NaCl, 3 mM KCl, 2 mM KPO_4, 10 mM $NaPO_4$, pH 7.4) and then fixed with 2% paraformaldehyde/PBS at room temperature for 15 min, permeabilized with 1% Triton X-100/PBS for 10 min, and incubated with 100 nM rhodamine−phalloidin in PBS in the dark for 30 min at room temperature. There were 3 × 5 min washes with PBS between each step. Coverslips were then inverted on a drop of Vectashield mounting media containing 1.5 μg/mL DAPI on a glass slide. Actin structures were visualized using Leica DM IRE2 confocal microscope system (Leica, Wetzlar, Germany) with excitation/emission wavelengths 360/450+/−20 nm for DAPI and 535/585+/−20 nm for rhodamine.

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Supporting Information Available. MALDI-TOF MS spectra of five representative beads, SPR analysis of the interaction between the tensin SH2 domain and immobilized pY peptides, inhibition of cell growth by Grb2 inhibitors, and effects of peptide treatment on cytoskeletal structure. This material is available free of charge via the Internet at http://pubs.acs.org.

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