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Structure-Based Design of Thioether-Bridged Cyclic Phosphopeptides Binding to Grb2-SH2 Domain

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Abstract—A series of phosphotyrosine containing cyclic peptides was designed and synthesized based upon the phage library derived cyclopeptide, **G1TE**. Considering the type-I β-turn feature of peptidic ligand binding to Grb2 SH2 domain, we introduce α,α-disubstituted cyclic amino acid, Ach, into the 4th position of the cyclic peptide to induce a local right handed 3_{10} helical conformation. In order to stabilize the favorable binding conformation, the bulky and hydrophobic amino acids, neopentylglycine (NPG) and phenylalanine, were introduced into the 8th and 2nd positions of the peptide ligand, respectively. To facilitate the sidechain of pTyr3 reaching into the phosphotyrosine binding pocket, a less bulky alanine was preferred in position 1. Based upon these global modifications, a highly potent peptide ligand **12** was discovered with an $IC_{50} = 1.68$ nM, evaluated by ELISA binding essay. Ligand **12** is at least 10^5 more potent than the lead peptide, termed **G1TE**.

Growth factor receptor-bound protein 2 (Grb2) is an essential intracellular adapter protein, consisting of one SH2 domain flanked by two SH3 domains.[1] Grb2-SH2 domain mediates intracellular signaling by binding to pTyr motifs on growth factor receptors such as EGFR and erbB2, providing a key link in the activation of mitogenic Ras pathways. Design of molecules that can selectively inhibit the association of EGF family of receptors, with the SH2 domain of the adapter protein, Grb2, to modulate the Ras signaling pathway. These agents may act as therapeutic leads for diseases, such as cancer, in which the Ras signaling pathway plays a major role.[2]

Unlike other SH2 domains of adapter proteins, the Grb2-SH2 domain is unique, in that the phosphopeptide ligands that bind it are required to adopt a β-turn conformation, which has been demonstrated by both NMR and X-ray analysis.[3–5] This characteristic binding feature makes the Grb2-SH2 domain an ideal target for drug development.[6-8]

In our previous studies, we discovered a novel disulfide-bridged cyclic peptide G1, generated by phage library.[9] This peptide was comprised of the sequence-Glu-Leu-Tyr-Glu-Asn-Val-Gly-Met-Tyr-, bracketed by two terminal cysteines that formed the cyclic peptide through a disulfide linkage. Subsequently, we further designed and synthesized its redox stable thioether-bridged analogue, cyclo (CH_2CO-Glu^1-Leu^2-Tyr^3-Glu^4-Asn^5-Val^6-Gly^7-Met^8-Tyr^9-Cys^{10})-amide, termed **G1TE**, which has shown comparable binding affinities ($IC_{50} = 15$–20 μM) to the Grb2-SH2 domain protein, in Biacore binding assays.[9] Notably, only the thioether analogue inhibited the ErbB2 receptor association with the Grb2 protein in tumor cell homogenates.

Compared to the linear phosphotyrosine-containing ligands, such as BCR-abl(pY177), EGFR(pY1068), erbB2(pY1139), and other synthetic ligands, **G1TE** and its analogues may form a larger circle-like binding surface when they bind to Grb2-SH2 domain, as suggested by molecular modeling and NMR studies.[10,11] Systematic alanine substitution of the amino acid components in synthetic analogues indicated that essentially all amino acids were required for sustenance of the biological activity, except Leu^2 and Gly^7.[10] The multipoint-binding feature of G1TE analogues provides for

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moderately potent agents, even without the presence of a phosphotyrosyl group. This binding pattern also provides us a unique opportunity and potential to further enhance their activity by rational molecular design. In this article, we would like to report our recent discovery of potent tyrosine-phosphorylated G1TE analogues by overall side-chain and backbone modifications and conformational constraint (Fig. 1).

Synthesis

The tyrosine phosphorylated peptide ligands were synthesized by using the procedure described in Figure 2. The linear peptide precursors were synthesized on an ABI 433A peptide synthesizer, starting with Fmoc-PAL resin for establishing the C-terminal carboxamide, and using the chemical protocols based on the Fmoc chemistry. The incorporation of N^{α}-Fmoc-Tyr[PO(OH, OBzl]-OH was accomplished manually by double coupling using HATU as the coupling reagent. All peptides were purified to homogeneity by RP-HPLC, and their structures were confirmed by FAB and/or MALDI mass spectrometry.

ELISA Assay

An N-terminally biotinylated phosphopeptide, DDPSpYVNVQ, encompassing a Grb2 SH2 domain binding sequence derived from SHC protein, was bound at 20 ng/mL to 96-well plates overnight. Nonspecific interactions were inhibited by blocking with 5% bovine serum albumin containing TBS. Recombinant purified Grb2 SH2-GST fusion protein and samples with serial dilutions were incubated for at least 2 h. After extensive washing with 0.1% bovine serum albumin in TBS, bound Grb2 SH2 domain was detected using anti-GST antibodies and goat anti-mouse antibody conjugated to alkaline phosphatase. Quantitation of bound alkaline phosphatase was achieved by a colorimetric reaction employing *para*-nitrophenyl phosphate as substrate. Each compound is subjected to a minimum of three determinations. Assays are conducted with a positive control of known potency, with additional controls being performed without peptide or protein.

Molecular Modeling

Simulations were performed with the Insight II 2000/Discover 97 modeling package, with the cff91 forcefield. Models were constructed based on our structure for the cyclic peptide G1TE, which was built, as described previously,[7] from the X-ray crystal structure of the KPFpYVNV peptide bound to the Grb2-SH2 domain.[3] For refinement, the peptide–receptor complex first underwent 50 steps of steepest descent and 150 steps of conjugate gradient minimization. Harmonic positional restraints on the backbone atoms were gradually relaxed over the course of the minimization to eliminate any steric clashes in the sidechains without inducing deformations in the backbone. Next, the complex was solvated with a layer of water molecules 25 Å thick over the binding site area, and equilibrated briefly with 10 molecular dynamics runs of 200 steps each at 300 K. Velocities were reassigned to a random Boltzmann distribution for each run. During dynamics all atoms were held fixed except for the peptide, binding site residues within 5 Å of the peptide, and the inner 15 Å of the water layer. The final structure was then minimized for 300 steps with the conjugate gradient algorithm.

Results and Discussion

It has been well documented that phosphotyrosine is important for linear peptide ligand binding to Grb2-SH2 domain by forming extensive hydrogen bonds and hydrophobic interactions.[3,12–14] Phosphorylation of Tyr^3 of **G1TE** provided for us peptide **1** that competitively bound to the Grb2 SH2 domain protein with an IC_{50} of 137 nM, in ELISA binding assays. This observation suggested that the G1TE cyclic peptide platform

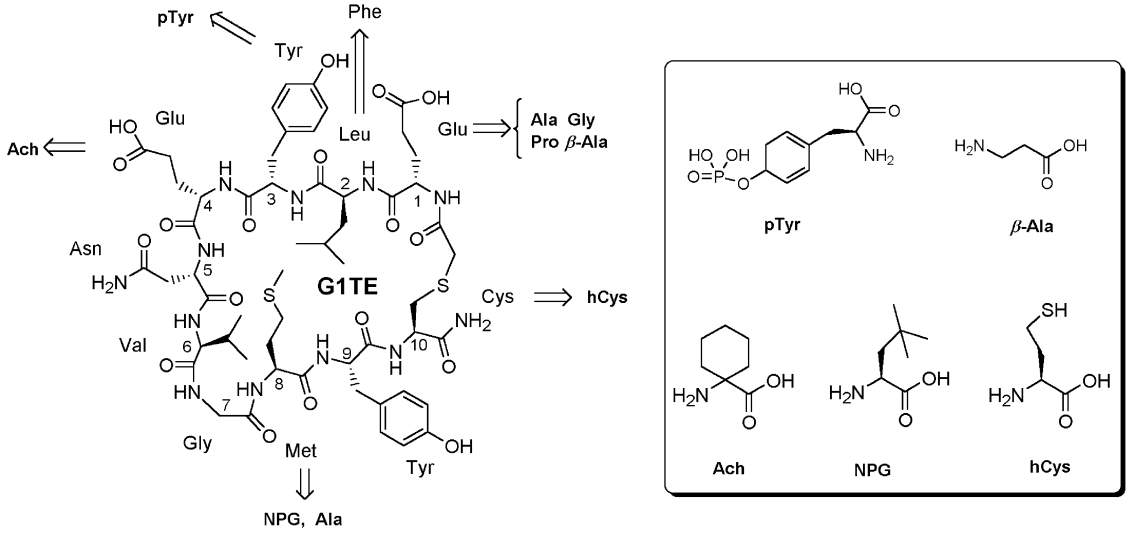

Figure 1. Molecular design of Grb2-SH2 domain antagonists based upon thioether-bridged cyclic peptide G1TE.

is also compatible with high affinity binding of pTyr-containing analogues. The inhibitory activity of pTyr-containing peptide **1** is at least 1000 times more potent than the corresponding non-phosphorylated analogue **G1TE**. Previously, we have found that amino acid in the Glu1 position is very sensitive to alterations in the non-phosphotyrosine containing analogues. However, for the tyrosine-phosphorylated derivatives, we observed that the functional role of the amino acid Glu^1 has become less significant. Replacement of Glu^1 with Pro, Gly, or Ala, does not change markedly the inhibitory activity of these pTyr-containing peptides. Interestingly, the acidic side-chain of Glu1 is disfavored in the pTyr-containing analogues, while the less bulky alanine is more favorable in this position. As shown in Table 1,

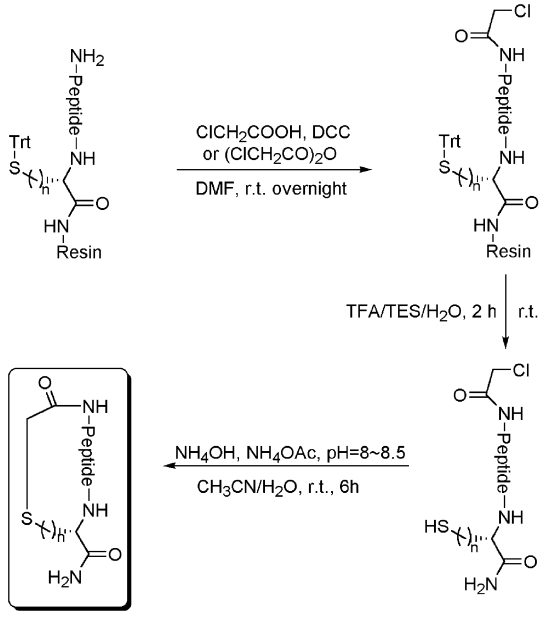

Figure 2. General procedure for the synthesis of thioether-bridged G1TE analogues.

Table 1. Inhibitory activities of phosphorylated Grb2-SH2 domain antagonists[a]

Compd	Amino acid composition						Activity IC_{50} (nM)[b]
	AA1	AA2	AA3	AA4	AA8	AA10	
G1TE	Glu	Leu	Tyr	Glu	Met	Cys	>100000[c]
1	Glu	Leu	pTyr	Glu	Met	Cys	137 (±53)
2	Gly	Leu	pTyr	Glu	Met	Cys	31.5 (±1.6)
3	Pro	Leu	pTyr	Glu	Met	Cys	75.7 (±5.0)
4	Ala	Leu	pTyr	Glu	Met	Cys	25.3 (±8.7)
5	Ala	Leu	pTyr	Glu	Met	hCys	54.7 (±5.4)
6	β-Ala	Leu	pTyr	Glu	Met	Cys	8.26 (±1.5)
7	β-Ala	Leu	pTyr	Ach	Met	Cys	21.0 (±5.4)
8	Ala	Leu	pTyr	Ach	Met	Cys	3.77 (±0.13)
9	Ala	Leu	pTyr	Ach	Ala	Cys	13.3 (±3.9)
10	Ala	Leu	pTyr	Glu	NPG	Cys	14.5 (±2.8)
11	Pro	Leu	pTyr	Ach	NPG	Cys	7.65 (±0.79)
12	Ala	Phe	pTyr	Ach	NPG	Cys	1.68 (±0.95)

[a]The binding affinity of these compounds was evaluated by ELISA assays.
[b]Values are means of three experiments, standard deviation is given in parentheses.
[c]Biacore assay indicates that G1TE exhibits inhibitory activity with a $IC_{50}=20$ μM,[7] and repetitive measurements give a range of 13–75 μM.[15]

the peptide **4** is 5-fold more potent than peptide **1**. This trend is the opposite to the trend we observed in the SAR studies of non-phosphotyrosine containing G1TE analogues.[10] For this reason, we studied in detail the binding pattern of pTyr-containing G1TE analogues to Grb2-SH2 domain. As shown in Figure 3, the phosphate group of pTyr in peptide **4** forms extensive hydrogen bonds with Arg^{67}, Arg^{86}, Ser^{88}, Ser^{90} and Ser^{96} of the Grb2-SH2 domain (Fig. 3a). While for the peptide ligand **1**, both the Glu^1 and pTyr side-chains need to be accommodated in the same conformational space, with the consequence of enlarging the binding pocket. This results in the loss of important binding interactions of the phosphate group with Arg^{67} and Ser^{90} (Fig. 3b). Although the acidic sidechain of Glu^1 can partially compensate for the loss of binding energy by forming several hydrogen bonds with the Grb^2 protein, the overall hydrogen bond network is still weaker than that in peptide **4**. In addition, the side-chain-flexible glutamic acid is less favored than alanine, considering the entropic penalty in the process of binding. Unexpectedly, when Glu^1 was replaced with β-Alanine, the resulting peptide **6** was 16 times more potent than peptide **1**. The enlargement of the peptide backbone ring size improves binding affinity by 4-fold when comparing peptide **2** with peptide **6**. On the contrary, if we enlarge the peptide backbone ring size in another location by replacing Cys^{10} with homocysteine, the Cys^{10} mutated compound **5** is 2-fold less active than the corresponding peptide **4**. The potentiating effect of β-alanine might be explained by virtue of the locally increased flexibility and conformational space of the peptide backbone near the phosphotyrosine residue that allows the pTyr to adopt a more optimal conformation to form favored hydrogen bond network within the pTyr binding pocket.

The X-ray structure of Grb2-SH2 domain complexed with pTyr-containing peptide ligand has shown that the phosphopeptide binds to Grb2-SH2 domain in a β-turn conformation due to the existence of Trp^{121} around the asparagine binding pocket.[3] Therefore, we intentionally introduced a turn-inducing amino acid, such as 1-amino-1-cyclohexylic acid (Ach),[13] in the position 4. We found that the Ach^4-containing peptide ligand **8** is approx. 6 times more potent than the corresponding peptide **4**. Molecular modeling indicates that Ach^4 indeed generates a short right-handed 3_{10} helix between $pTyr^3$ and Asn^5, with the torsion angles $\Phi=-52.5°$, $\Psi=-48.8°$ and $\omega=175°$ (Fig. 4). This turn conformation greatly benefits the binding of the peptide ligand to the characteristic binding pocket of the Grb2-SH2 domain. The hydrophobic Van der Waals interactions of Ach sidechain with Gln^{106} and Phe^{108} is also a positive factor towards enhancement of binding affinity of Ach^4 containing peptide, as shown in Figure 4. Moreover, this Ach induced turn may globally influence the backbone conformation, and the enlargement of backbone ring in position 1 is not favored under this circumstance (Table 1, peptide **7** vs **6**).

To further enhance the inhibitory activity of these cyclic peptides, we also investigated the functional significance

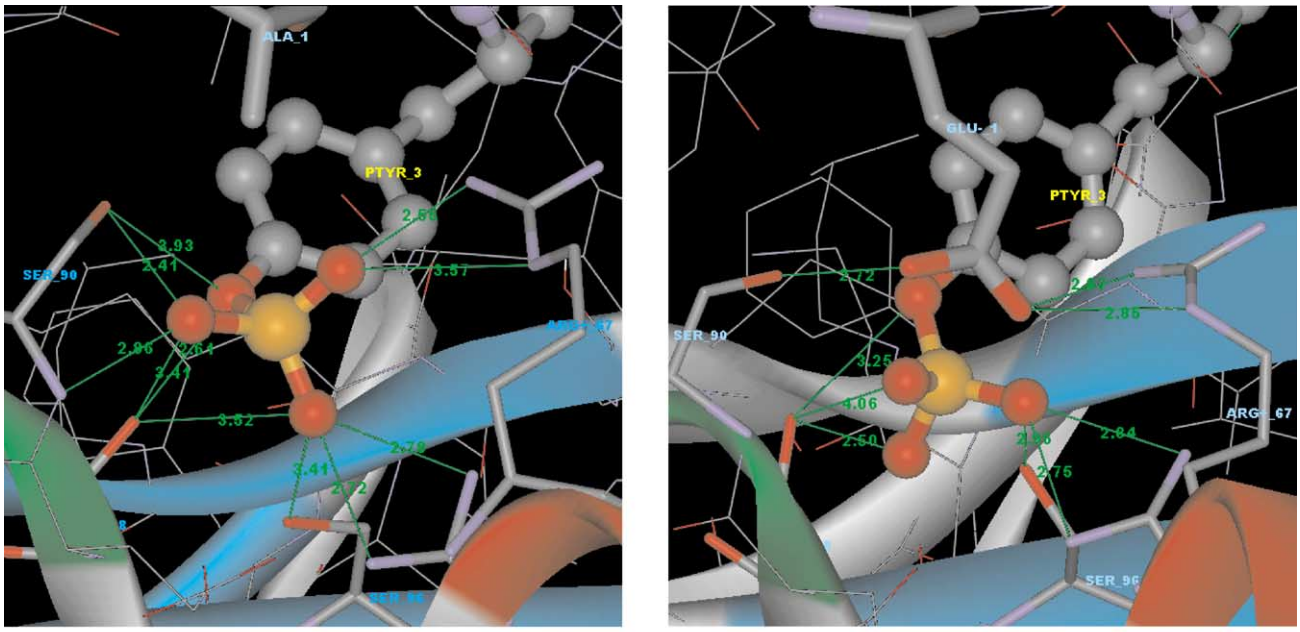

Figure 3. Interactions of peptide **1** (a) and **4** (b) with Grb2-SH2 domain within the phosphotyrosine binding pocket.

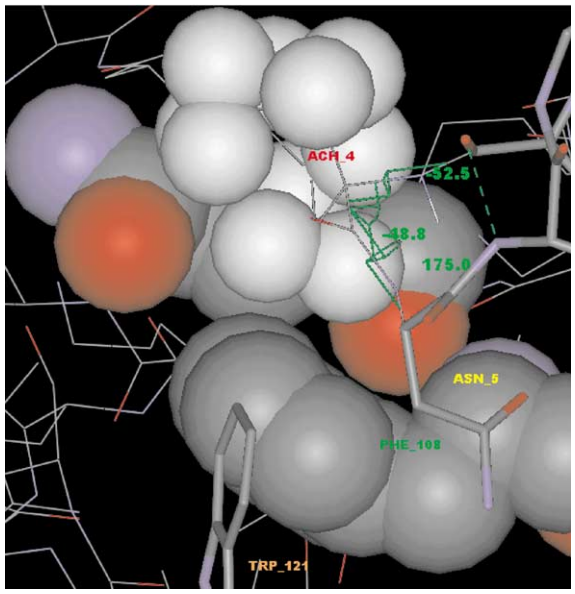

Figure 4. Right-handed 3_{10} helix of peptide **8** induced by Ach[4] and Van der Waals interactions of Ach[4] (the hydrogens of the side-chain presented in CPK style and the backbone presented in line style) with the Gln[106] and Phe[108] (presented in CPK style) of Grb[2] protein.

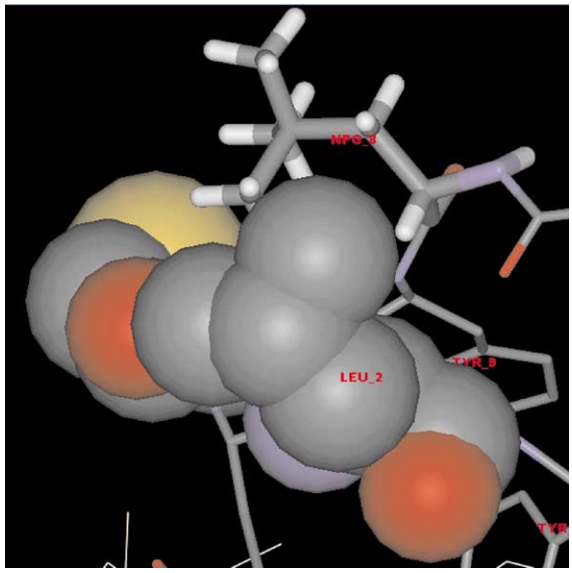

Figure 5. Hydrophobic packing of NPG[8] (presented in stick style) with Leu[2], Cys[10] and the *N*-acetyl group (presented in CPK style) in the cyclic peptide **10**.

of Met[8] since this amino acid residue is also very sensitive to mutation according to our previous work.[10] As shown in Table 1, the activity drops approx. 5-fold when Met[8] was replaced with Alanine (peptide **8** vs **9**). Notably, Met[8], Cys[10], and Leu[2] are found to have no interactions with the Grb[2] protein, but the sidechains of these amino acid residues form hydrophobic packing with each other to stabilize the favored conformation for other residues binding to Grb2-SH2 domain. If Met[8] is substituted with Alanine, this type of packing will be deficient, and will result in an increase of the flexibility of the sidechain and backbone of peptide **9**. Based upon this observation, we introduced the bulky and less flexible neopentylglycine (NPG) in position 8. The inhibitory activity of the modified peptide **10** was enhanced approx. 2-fold in comparison with peptide **4**, due to the well organized packing of NPG[8], Cys[10], Leu[2] and the *N*-acetyl group within the peptide ligand **10** (Fig. 5). By replacing the flexible Leu[2] with relatively more rigid and electron-rich phenylalanine, we can further optimize this

hydrophobic packing. Thus, we obtained the high affinity binding peptide **12** with an $IC_{50} = 1.68$ nM, based upon ELISA assays. This SAR information provides us useful guide for the further development of conformationally constrained and highly potent peptide-derived ligands binding to Grb2-SH2 domain.

Conclusion

Based upon the phage library derived cyclic peptide **G1TE**, we designed and synthesized a series of phosphotyrosine containing cyclic peptides guided by molecular modeling. By introducing a less bulky alanine in the 1st position, a turn-inducing Ach in 4th position, a hydrophobic and less flexible NPG and phenylalanine in the 8th and 2nd positions, respectively, we developed a globally and locally optimized inhibitor **12** with a $IC_{50} = 1.68$ nM, as evaluated by ELISA assays. This highly potent peptide ligand provides us an ideal lead compound for the development of pharmacokinetically improved peptidomimetics that can selectively bind to Grb2-SH2 domain, and act as potential therapeutic agents for the treatment of erbB2-overexpressed breast cancer and other diseases.

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