Communications to the Editor

Potent Antagonists of the SH2 Domain of Grb2: Optimization of the X_{+1} Position of 3-Amino-Z-Tyr(PO$_3$H$_2$)-X_{+1}-Asn-NH$_2$

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A growing number of the elements identified in intracellular signaling events that affect cell growth and transformation are proteins that physically interact with each other via domains or specifically recognized amino acid sequences. These intracellular protein–protein interactions are particularly attractive targets in oncology due to their participation in controlling mitogenic signal transduction pathways that are activated or deregulated in cancer cells.[1–3] This is the case for the protein–protein interactions involving the Src homology 2 (SH2) domain of Grb2[4] in the Ras signal transduction pathway.[5–7] In mammalian cells, the Grb2 adaptor protein links the tyrosine kinase receptors activated by growth factors to Sos, a guanine nucleotide exchange factor that, upon receptor binding and translocation of the Grb2–Sos complex to the plasma membrane, converts the inactive Ras·GDP to active Ras·GTP.[8] Activated Ras triggers the MAP kinase cascade that is essential for cell growth and differentation.[9] The interaction between the activated tyrosine kinase receptors and Grb2 is mediated by the Src homology 2 domain of the signaling protein. Agents that specifically disrupt this protein–protein interaction could potentially shut down the Ras pathway and present an intervention point for blocking human malignancy.

Starting with the minimal recognition motif of the SH2 domain of Grb2,[10] we have initiated a medicinal chemistry program to identify compounds that specifically disrupt the interaction between activated tyrosine

kinase receptors and the Grb2–SH2 domain. We recently reported the design of an N-terminal group that can impart high affinity to the minimal tripeptide sequence recognized by the Grb2–SH2 domain.[11] In the present communication, we report a further improvement in the binding affinity of the 3-amino-Z-Tyr(PO$_3$H$_2$)-X_{+1}-Asn-NH$_2$ phosphopeptide by optimizing the X_{+1} position.[12]
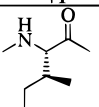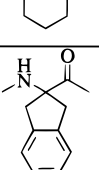
Degenerate phosphotyrosyl peptide libraries have shown that the sequence specificities of SH2 domains for phosphotyrosyl peptides lay in the portion of the peptide immediately carboxy-terminal of the phosphotyrosine residue.[13,14] For the SH2 domain of Grb2, the consensus sequence is Tyr(PO$_3$H$_2$)-X_{+1}-Asn-X_{+3} and the residue that determines specificity is asparagine.[13,15] Synthetic[16] and phage display library[17] approaches have been used to identify optimal residues carboxy-terminal to phosphotyrosine. Although these studies agree on the exclusive selectivity for asparagine at the X_{+2} position, differences between the two techniques were observed for positions X_{+1} and X_{+3}. For position X_{+1}, glutamine, glutamic acid, isoleucine, tyrosine, and valine were selected from synthetic peptide libraries,[16] while a preference for glutamic acid and methionine residues was identified by phage display.[17] However, selection of glutamic acid, glutamine, methionine, or valine at the X_{+1} position did not alter the binding affinity of phosphotyrosyl peptides for the Grb2–SH2 domain.[17] The above results seemed to indicate a low stringency for this position.

The unique structural features of the ligand-bound Grb2–SH2[18] were exploited to optimize the X_{+1} position of 3-amino-Z-Tyr(PO$_3$H$_2$)-X_{+1}-Asn-NH$_2$, a recently reported potent antagonist of the Grb2–SH2 domain (X_{+1} = Ile).[11]

The structure of the Grb2–SH2 domain complexed with a 7-mer phosphotyrosyl peptide[19] derived from the BCR-abl protein was determined in our group by X-ray crystallography at 2.1 Å resolution.[18] The folding of the SH2 domain of Grb2 shows a general pattern consisting of a central antiparallel β-sheet flanked by two α-helices, but the ligand has a folded conformation, in contrast to
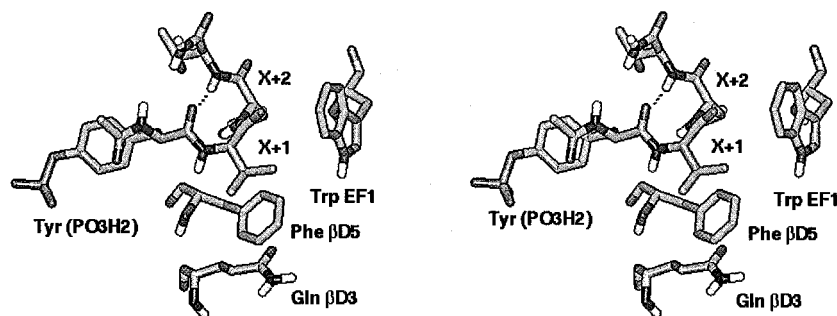
Table 1. Structure−Activity Relationships of Phosphopeptides with the General Sequence 3-Amino-Z-Tyr(PO$_3$H$_2$)-X$_{+1}$-Asn-NH$_2$[a]

Entry	X$_{+1}$	IC$_{50}$ (nM)
1		65 ± 5
2		206 ± 14
3		124 ± 6
4		23 ± 1
5		1 ± 0.2
6		140 ± 5

[a] Competitive binding assays with the recombinant SH2 domain of Grb2 expressed as a glutathione S-transferase fusion protein and the immbolized tyrosine-phosphorylated MPB-EGFR were conducted as previously described.[11,26] Dose−response relationships were constructed by nonlinear regression of the competion curves with GraFit 3.0 (Erithacus Software Limited, London, U.K.). The errors quoted correspond to the standard error in the fits of the data.
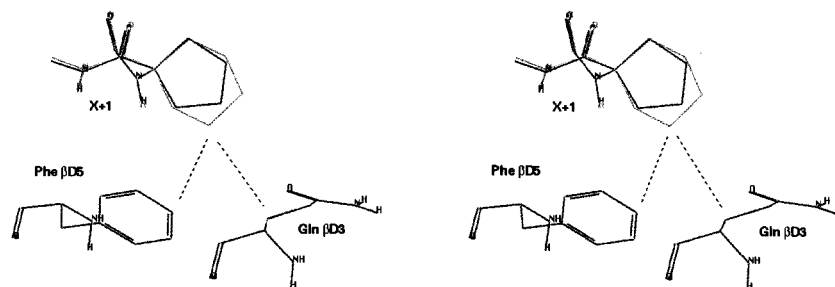
all previously reported structures where the phosphotyrosyl peptide adopts an extended conformation.[20] The Grb2−SH2 domain has a bulky tryptophan residue (Trp-121) in position 1 of the EF loop. The side chain of this amino acid closes the binding cleft C-terminal to phosphotyrosine and forces the ligand to adopt a type I β-turn conformation (Figure 1). This conformation is maintained by a hydrogen bond between the carbonyl group of phosphotyrosine and the backbone nitrogen of the residue at the X$_{+3}$ position. Due to this type I β-turn conformation, the residue at the X$_{+1}$ position presents a local right-handed 3$_{10}$ helical conformation. We posited that improved potency could be obtained by incorporating α,α-dialkylated amino acids at the X$_{+1}$ position of phosphopeptide antagonists of the Grb2−SH2 domain; α,α-disubstituted amino acids are known to stabilize α and 3$_{10}$ helices in short peptide motifs,[21−23] and the incorporation of such building blocks at the X$_{+1}$ position of 3-amino-Z-Tyr(PO$_3$H$_2$)-X$_{+1}$-Asn-NH$_2$ should favor the adoption of a conformation close to the one observed in the ligated form of the phosphopeptide. Additional structural information was taken into consideration for selection of the α,α-disubstituted linear and cyclic amino acids. In the X-ray structure of the ligand-bound Grb2−SH2, the side chain of valine at the X$_{+1}$ position of the phosphopeptide ligand makes six van der Waals contacts[24] with PheβD5 and one van der Waals interaction with GlnβD3[25] (Figure 1). Therefore, the α,α-disubstituted amino acid at the X$_{+1}$ position should not only induce a local right-handed 3$_{10}$ helical conformation, but its side chain should also mimic the above hydrophobic interactions. Table 1 shows some selected examples of the SAR data obtained. Two building blocks, 1-aminocyclopentanecarboxylic acid (**4**) and 1-aminocyclohexanecarboxylic acid (**5**), showed respectively a 3-fold and 65-fold increase in binding affinity relative to **1**, our reference compound. The 1-aminocyclopentanecarboxylic acid derivative (**4**) is less active than its six-membered ring analogue (**5**), probably because in the former the Cγ atom of the ring,[27] which corresponds to one of the Cγ atoms of the valine residue in the X-ray structure of the ligand-bound Grb2−SH2 domain (Figure 1), is not well positioned and forms weaker van der Waals interactions with PheβD5 and GlnβD3 (Figure 2). To further illustrate the importance of the van der Waals contacts in keeping the binding affinities in the low nanomolar range, we have included in Table 1 the data obtained with α-aminoisobutyric acid (**2**) and 1-aminocyclopropanecarboxylic acid (**3**). Even if these building blocks are able to induce the adoption

Figure 1. Conformation of the C-terminal part of the phosphopeptide ligand[19] in the X-ray structure of the ligated Grb2−SH2 domain.[18] The intramolecular hydrogen bond formed between the carbonyl group of phosphotyrosine and the backbone nitrogen of the residue at the X$_{+3}$ position appears as a dashed line.

Table 2. Relative Affinities (IC$_{50}$) of Phosphopeptide **5** for GST/Grb2, Lck, p85, and Shp2 SH2 Domains and SHC PTB Domain[a]

	Grb2 SH2	Lck SH2	p85 SH2[b]	Shp2 SH2	SHC PTB
sequences	Biotin-XnpYINQXn	Biotin-XnpYEEIXn	Biotin-XnpYVPMXn	Biotin-XnpYTAVXn	Biotin-XnENPQpYXn
peptide **5**	0.004 ± 0.001	6.24 ± 0.39	0.97 ± 0.03	>5	>5

[a] The results are expressed as the concentration at which half-maximal competition was observed (IC$_{50}$, μM). Competitive binding phosphopeptide assays were conducted as previously described.[31] The errors quoted correspond to the standard error in the fits of the data. [b] N-Terminal SH2 domain.

Figure 2. Superimposed energy minimized models (MacroModel v.4.0[28]) of compounds **4** and **5** showing the side chains of 1-aminocyclopentanecarboxylic acid (black) and 1-aminocyclohexanecarboxylic acid (gray) at the X_{+1} position of the above peptides. The van der Waals interactions (3.4 Å $\leq d \leq$ 4 Å) involving the C^γ atom of the cyclohexyl ring and the side chains of PheβD5 and GlnβD3 appear as dashed lines.

of a favorable conformation, they cannot establish the hydrophobic interactions observed in the X-ray structure of the ligand-bound Grb2−SH2 domain. The incorporation of 2-aminoindan-2-carboxylic acid (**6**) at the X_{+1} position represents an attempt to create an additional van der Waals interaction with PheβD5 or GlnβD3, depending on the conformation of the unsaturated ring. Although modeling studies suggested that there is enough space at the surface of the protein to accommodate the additional aromatic ring, no gain in activity was observed (Table 1). On the contrary, **6** is significantly less active than its parent compound **4** (Table 1). A hydrophobic collapse[29] of the bulky amino acid at the X_{+1} position with the N-terminal moiety of the phosphopeptide, resulting in an unfavorable preorganized conformation of the antagonist in its unligated state, could explain the drop in binding activity observed for **6**.[30]

Phosphopeptide **5** is not only a very potent antagonist of the Grb2−SH2 domain but also very selective for this SH2 domain. Table 2 presents the data obtained in competitive binding assays with phosphopeptide **5** and different SH2 domains. The phosphopeptide shows a 243−1560-fold preferential binding to Grb2−SH2 over p85 N-terminal SH2 and Lck SH2, respectively, and at least 1250-fold selectivity to Grb2−SH2 over Shp2 SH2 and SHC PTB.

In summary, the unique structural features of the SH2 domain of Grb2 have been exploited to optimize the X_{+1} position of 3-amino-Z-Tyr(PO$_3$H$_2$)-X_{+1}-Asn-NH$_2$. Phosphopeptide **5** is, to the best of our knowledge, the most potent antagonist so far reported for the SH2 domain of Grb2. Other examples of the use of structural information to increase the binding affinity of antagonists of the Grb2−SH2 domain will be reported from our group in due course.

Supporting Information Available: Synthesis and analytical data for compounds **1**−**6** and a description of the biological assays (6 pages). Ordering information is given on any current masthead page.

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