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Mapping the X_{+1} binding site of the Grb2-SH2 domain with α,α-disubstituted cyclic α-amino acids

Carlos García-Echeverría,*[,1] Brigitte Gay,[1] Joseph Rahuel[2] and Pascal Furet[1]

Novartis Pharma Inc., [1]Oncology Research Department and [2]Core Technology Area, 4002 Basel, Switzerland

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Abstract. A series of phosphopeptides containing α,α-disubstituted cyclic α-amino acids (Ac_nc, $3 \leq n \leq 7$; n refers to the number of carbons in the ring) at the X_{+1} position of Ac-Tyr(PO_3H_2)-X_{+1}-Asn-NH_2 has been synthesised and their inhibitory activity as antagonists of the Grb2-SH2 domain has been determined in competitive binding assays. The SAR data obtained have been interpreted by using models constructed from the X-ray structure of the ligand-bound Grb2-SH2 domain. The used of α,α-disubstituted cyclic α-amino acids to map the binding pockets of proteins expands the classical alanine scan concept and takes advantage of the known conformational preferences of these amino acids.

Introduction. Our group has previously determined the X-ray structure of the Grb2-SH2 domain complexed with a phosphotyrosyl peptide derived from an endogenous ligand.[1] The folding of the SH2 domain of Grb2 shows a general pattern, but the ligand adopts a type I β-turn conformation, in contrast to all previously reported ligand-bound SH2 domain structures in which the phosphotyrosyl peptide has an extended conformation. We have recently shown how this unique structural feature can be exploited to increase the binding affinity of antagonists of the Grb2-SH2 domain.[2] Our approach involved the use of α,α-disubstituted cyclic α-amino acids to stabilize the 3_{10} helical backbone conformation required at position i +1 of the β–turn.[3] We have now complemented and extended our previous work by a systematic structure-activity study in which each α,α-disubstituted cyclic α-amino acid in the series Ac_nc with $3 \leq n \leq 7$ was incorporated at the X_{+1} position of the minimal recognition motif of the Grb2-SH2 domain, Ac-Tyr(PO_3H_2)-X_{+1}-Asn-NH_2. The results of this study further delineate the scope and utility of α,α-disubstituted cyclic α-amino acids as valuable tools in the mapping of binding sites of proteins.

Synthesis. The phosphopeptides of Table 1 were synthesized manually starting with a 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin[4] for establishing the required *C*-terminal carboxamide and using standard-solid phase protocols based on fluorenylmethoxycarbonyl (Fmoc) chemistry.[5] Ninhydrin negative end-points for the coupling of the α,α-disubstituted cyclic α-amino acids were reached by using

* To whom correspondence should be addressed. E-mail: carlos.garcia-echeverria@pharma.novartis.com and pascal.furet@pharma.novartis.com. Fax: 41 61 696 62 46.

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benzotriazolyloxy-tris-(dimethylamino)phosphonium hexafluorophosphate/1-hydroxybenzotriazole[6] (1:1) as coupling agent. N^{α}-Fmoc-Tyr(PO_3H_2)-OH[7] was incorporated with benzotriazolyloxy-tris-(dimethylamino)phosphonium hexafluorophosphate/1-hydroxybenzotriazole[6] (1:1; first coupling) and *N*-[(dimethylamino)1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide[8] (second coupling) in the presence of diisopropylethylamine. The completed peptide resins were simultaneously deprotected and cleaved by treatment with trifluoroacetic acid-water (19:1, v/v) for 3 h at room temperature. The filtrate from each cleavage reaction was added to diisopropyl ether-petroleum ether (1:1, v/v) at 0° C, and the resulting precipitate was collected by filtration. The crude peptides were purified by medium-pressure liquid chromatography on a C_{18}-column using an acetonitrile-water gradient. The purity of the final compounds was verified by reversed-phase analytical HPLC and the identity was assessed by correct mass spectral analyses.[9]

Results and Discussion. The inhibitory activities of the phosphopeptides containing the Ac_nc ($3 \leq n \leq 7$) amino acids at the X_{+1} position are reported in Table.1. These values were determined in our EGFR (Epidermal Growth Factor Receptor) assay, which measures the potency of a compound to inhibit the binding of the phosphorylated intracellular domain of EGFR to the Grb2-SH2 domain.[10] The activities of two reference phosphopeptides (**6** and **7**, Table 1) are also included.

Table 1. Inhibitory activity of phosphopeptides with the general sequence Ac-Tyr(PO_3H_2)-Ac_nc-Asn-NH_2 ($3 \leq n \leq 7$) and reference phosphopeptides Ac-Tyr(PO_3H_2)-X_{+1}-Asn-NH_2 (X_{+1} = Gly, Val).[a]

Entry	1	2	3	4	5	6	7
Ac_nc	Ac_3c	Ac_4c	Ac_5c	Ac_6c	Ac_7c	Gly[b]	Val[b]
IC_{50} (μM)	72.5 ± 5.4	18.2 ± 0.4	7.9 ± 0.8	0.21 ± 0.02	1.11 ± 0.12	67.0 ± 8.4	4.3 ± 0.9

[a]Competitive binding assays with the recombinant SH2 domain of Grb2 expressed as a glutathione S-transferase fusion protein and the immobilized tyrosine-phosphorylated maltose binding protein-EFGR were conducted as previously described.[10] Dose-response relationships were conducted 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.
[b] Values taken from ref. 11.

As can be seen in Table 1, phosphopeptide **1** (X_{+1}= Ac_3c) has roughly the same potency as the reference phosphopeptide **6** (X_{+1}= Gly), which cannot engage in side chain interactions with the X_{+1} binding site of the protein. There is a continuous increase in potency going from phosphopeptide **1** (X_{+1}= Ac_3c) to **4** (X_{+1}= Ac_6c). This trend ends with phosphopeptide **5** (X_{+1}= Ac_7c), which is one order of magnitude less potent than **4** that represents the most potent antagonist in this series. Besides the expected effect of promoting a local helical backbone conformation, the incorporation of an α,α-disubstituted cyclic α-amino acid at the X_{+1} position of the minimal recognition motif of Grb2-SH2 has implications in terms of ligand-protein interactions. To interpret the structure-activity relationships of Table 1 on the basis of these two complementary effects, models

of phosphopeptides **1-5** bound to the Grb2-SH2 domain were constructed using available structural information.[12] These models allowed us to analyze in detail the possible hydrophobic contacts of the cyclic side-chain of the Ac_nc ($3 \leq n \leq 7$) amino acids with the X_{+1} binding site of Grb2-SH2.

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 seven favorable hydrophobic van der Waals contacts with Phe βD5 and Gln βD3.[13] These interactions involve the Cβ atom – three van der Waals contacts – as well as the two Cγ atoms – two interactions each – of valine. All of the Ac_nc amino acids included in this study possess a Cβ atom and for $n \geq 4$ they also have a Cγ atom that geometrically corresponds to either one of the Cγ atoms of valine. Compared to valine in the X-ray structure of the ligand-bound Grb2-SH2, 1-aminocyclopropanecarboxylic acid (entry **1**) should be able to make only three hydrophobic contacts with Phe βD5 and Gln βD3, while in principle it should be possible for the other Ac_nc residues ($n \geq 4$; entries **2-5**) to establish five van der Waals interactions. However, due to the geometrical constraints imposed by the cyclic side chain not all of these interactions can effectively be realized. In accordance with the data reported in Table 2, the models suggest that the Ac_nc amino acids with $3 \leq n \leq 5$ (entries **1-3**) make less contacts than expected because the strained geometry of their cyclic side chain moves the atoms away from the Grb2-SH2 domain. In contrast, the Ac_nc amino acids with n= 6 or 7 (entries **4** and **5**) are able to establish additional contacts because their Cγ atoms are closer to Phe βD5 and Gln βD3 (Figure 1). On the other hand, the model for the Ac_7c-containing peptide also points to a short repulsive contact (interatomic distance less than 3.0 Å) between one of the Cδ atoms of the ring and the oxygen atom of the side chain carbonyl group of Gln βD3 (Figure 1).

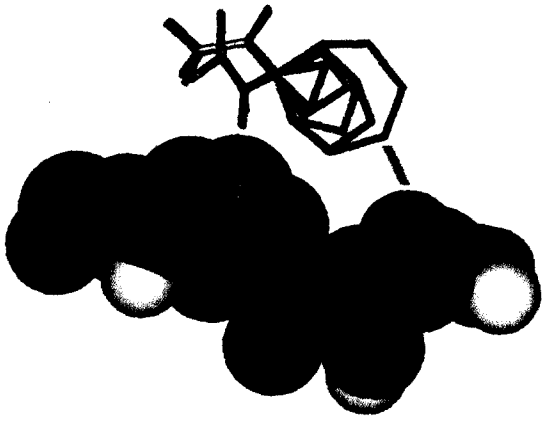

Figure 1. Models of the Ac_nc amino acids ($3 \leq n \leq 7$) at the X_{+1} binding site of the Grb2-SH2 domain (n=3 red, n=4 blue, n=5 grey, n=6 pink and n=7 yellow). The short contact between the Cδ atom of the side chain of Ac_7c and the carbonyl oxygen of Gln βD3 is represented as a dashed line.

Table 2. Number of van der Waals contacts[a] observed in the models of Figure 1 between the amino acid at the X_{+1} position and the Grb2-SH2 domain residues Phe βD5 and Gln βD3.

X_{+1}	Ac_3c	Ac_4c	Ac_5c	Ac_6c	Ac_7c	Gly	Val
n° of van der Waals contacts	2	2	4	8	8	0	7

[a]A hydrophobic van der Waals contact exists when the carbon-carbon interatomic distance between the ligand and the protein ranges from 3.4 to 4.2 Å [14,15]

Based on the constructed models, the structure-activity relationships presented in Table 1 can be rationalized as follows. Although two favorable hydrophobic contacts are created when glycine (entry **6**) is replaced by 1-aminocyclopropanecarboxylic acid (entry **1**), this single amino acid mutation does not improve the binding affinity of the minimal recognition motif of Grb2-SH2 (Table 1). This implies a slight detrimental conformational effect that neutralizes the benefit obtained by creating the two new hydrophobic interactions. The known preference of 1-aminocyclopropane carboxylic acid[16] for the region of the conformational space corresponding to position i + 2 of a type I or II β-turn[17] rather than that corresponding to a 3_{10} helix[18] – the local conformation required here – is fully consistent with the present data. The beneficial effect of restricting the backbone conformation towards the 3_{10} helix region starts to take effect for the Ac_nc amino acids with $n \geq 4$. The positive conformational effect is most apparent when one compares the potencies of the phosphopeptides **3** (X_{+1}= Ac_5c) and **4** (X_{+1}= Ac_6c) to that of the reference phosphopeptide **7** (X_{+1}= Val). Although 1-aminocyclopentanecarboxylic acid makes three hydrophobic contacts less than valine (Table 2), phosphopeptides **3** and **7** have similar inhibitory activities (Table 1). Phosphopeptide **4**, which is the most potent antagonists in this series, is one order of magnitude more potent than **7** (20-fold increase in activity) although being involved in only one additional van der Waals contact (Table 2). The drop in activity observed for phosphopeptide **5** (X_{+1}= Ac_7c) when compared to **4** (X_{+1}= Ac_6c), which has the same number of van der Waals contacts (Table 2), can be ascribed to a potential repulsive interaction between the Cδ atom of the side chain of Ac_7c and the oxygen of the side chain of Gln βD3 (Figure 1). The side chain amide moiety of Gln βD3 can move to avoid this steric clash, but this motion would cause the loss of a hydrogen bond interaction with an adjacent residue in the protein.

Conclusions. The results reported in this letter illustrate the usefulness, but also some of the limitations of α,α-disubstituted cyclic α-amino acids as tools in the mapping of binding pockets of proteins.[19] Conformational preferences as well as the number of van der Waals contacts between the ligand and the protein have been considered in order to interpret the activity data. The improvement in affinity obtained when the ring size of the α,α–disubstituted cyclic α-amino acid at the X_{+1} position of the minimal recognition motif of the Grb2-SH2 domain was increased from four to six atoms clearly correlates with the number of additional

favorable contacts formed with the protein. This indicates that no major differences exist between these residues with regard to their propensity to promote a local 3_{10} helical conformation.

The model presented in this letter for the phosphopeptide containing the 1-aminocyclohexanecarboxylic acid residue has been in the meantime confirmed in our group by solving the X-ray structure of the Grb2-SH2 domain complexed with a ligand containing this residue at the X_{+1} position and an asparagine mimetic at the X_{+2} position.[20] The van der Waals interactions observed in the X-ray structure are in full agreement with the ones proposed and discussed above.

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References and Notes

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9) The purity of the phosphopeptides was verified by reversed-phase analytical HPLC on a Nucleosil C_{18}-column (250 x 4 mm, 5 µm, 100 Å): i) linear gradient over 10 min of MeCN/0.09% TFA and H_2O/0.1% TFA from 0:1 to 1:9, flow rate 2.0 mL/min, detection at 215 nm, single peak at t_R= 4.32 min (Ac_5c); t_R= 6.07 min (Ac_4c); ii) linear gradient over 10 min of MeCN/0.09% TFA and H_2O/0.1% TFA from 1:49 to 3:2, flow rate 2.0 mL/min, detection at 215 nm, single peak at t_R= 4.19 min (Ac_5c); t_R= 4.89 min (Ac_6c); t_R= 6.56 min (Ac_7c). ESI MS (negative ion mode) 498 $[M - H]^+$ (Ac_5c, calc. 498.4, $C_{19}H_{25}N_5O_9P_1$); 512

[M - H]$^+$ (Ac_4c, calc. 512.4, $C_{20}H_{27}N_5O_9P_1$). MALDI-TOF mass spectra (negative-ion mode): 526.5 (Ac_5c, calc. 526.5, $C_{21}H_{29}N_5O_9P_1$); 540.3 (Ac_6c, calc. 540.5, $C_{22}H_{31}N_5O_9P_1$); 554.0 (Ac_7c, calc. 554.5, $C_{23}H_{33}N_5O_9P_1$). The reference phosphopeptides **6** and **7** were reported in ref. 11.

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12) The models were obtained by replacing the X_{+1} valine in the ligand-bound SH2 domain X-ray structure (PDB code 1TZE) by the Ac_nc amino acids constructed (in right-handed 3_{10} helical conformations) using X-ray crystal structures of peptides containing these residues retrieved from the Cambridge Crystallographic Database. The database codes of the structures used to construct Ac_3c, Ac_4c, Ac_5c, Ac_6c and Ac_7c are respectively SIDWIL, AMCBCX, GIPJAQ, GARFUA and NIWCAX. The Ac_6c amino acid was built with the amino functionality adopting an axial position as observed by X-ray crystallography (Valle, G.; Crisma, M. Toniolo, C.; Sen, M.; Sukumar, M.; Balaram, P. *J. Chem. Soc. Perkin Trans. II* **1988**, 393). The ring of Ac_7c was given a twist-chair conformation since it is the most frequently observed in X-ray structures of peptides containing this amino acid (Benedetti, E.; Di Blasio, B.; Iacovino, R.; Menchise, V.; Saviano, M.; Pedone, C.; Bonora, G.M.; Ettorre, A.; Graci, L.; Formaggio, F.; Crisma, M.; Valle, G.; Toniolo, C. *J. Chem. Soc. Perkin Trans.* **1997**, *2*, 2023). No energy minimizations were performed since the geometries of the Ac_nc amino acids were taken from X-ray structures and the rest of the peptide was not modified. The models were constructed using Macromodel (Mohamadi, F.; Richards, N.G.; Guida, W.C.; Liskamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hendrickson, T.; Still, W.C. *J.Comp.Chem.* **1990**, *11*, 440-467).

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17) Dihedral angles for the position i + 2 of a type I or II β-turn: $\Phi = \pm 90°$, $\Psi = 0°$.

18) Dihedral angles for the position i + 1 of a type I β-turn: $\Phi = -60°$, $\Psi = -30°$.

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