Small Peptides Containing Phosphotyrosine and Adjacent αMe-Phosphotyrosine or Its Mimetics as Highly Potent Inhibitors of Grb2 SH2 Domain

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A series of small peptides with the sequence mAZ-pTyr-Xaa-Asn-NH_2, where Xaa denotes α-methylphosphotyrosine or its carboxylic mimetics, were synthesized as inhibitors of the Grb2 SH2 domain. Peptide **3** with (α-Me)pTyr as Xaa has the highest affinity for Grb2 (K_d = 3 ± 1 nM) and exhibits to date the best inhibitory activity (IC_{50} = 11 ± 1 nM) to displace PSpYVNVQN-Grb2 interaction in an ELISA test. The lower affinities of peptides with (α-Me)-Tyr, (α-Me)Phe(4-CO_2H), or (α-Me)Phe(4-CH_2CO_2H) as Xaa demonstrate the importance of a double charged phosphate group at the pY+1 position. Molecular modeling showed additional hydrogen bond interactions provided by the (α-Me)pTyr residue with the Grb2 SH2 domain. These results thus show that the effect of hydrophobic pY+1 residues, initially put forth to increased the binding affinities, can be further enhanced by a (-Me)pTyr residue which has both hydrophobic and hydrophilic properties.

Introduction

The adaptor protein Grb2, encompassing one SH2 and two SH3 domains,[1] is an important element in the signal transduction pathway which directs cell proliferation and differentiation.[2] In unstimulated mammalian cells, Grb2 exists in complexes with Sos. Upon cellular receptor activation by growth factors, the Grb2−Sos complex is translocated to the plasma membrane, owing to the direct binding of the SH2 domain to autophosphorylated receptors with endowed tyrosine kinase activity (RTK) such as EGF-R,[3] or via a phosphorylated adaptor protein Shc.[4] This translocation allows Sos to activate the Ras signaling pathway that is essential for cell growth and differentiation.

Anarchic cell proliferation, observed in some leukemias[5] and in breast and ovarian cancers,[6,7] was related to dysfunctioning of receptor or cytoplasmic proteins with tyrosine kinase activities coupled to p21 Ras activation. Thus, inhibition of protein−protein interactions in this pathway could provide an attractive approach in cancer therapy research.

To interrupt the Ras signaling pathway at the level of Grb2, we have recently designed peptidimers which can block simultaneously both SH3 domains of Grb2.[8] In this paper, we report the results of another approach which consists of novel ligands directed toward the Grb2 SH2 domain, as already reported by the group of Novartis Pharma Inc. Thus Rahuel et al. resolved, by X-ray crystallography, the structure of the Grb2 SH2 domain complexed with a phosphopeptide (KPFpYVNV), derived from the *BCR-Abl* oncogene, and observed that the complexed phosphopeptide adopts a type I β-turn conformation.[9] Starting from the minimal recognition motif of the Grb2 SH2 domain derived from the EGF-R, a small N-protected peptide mAZ-pTyr-Ac_6c-Asn-NH_2 (mAZ, *m*-aminobenzyloxycarbonyl; Ac_6c, 1-aminocyclohexanecarboxylic acid) that exhibits high binding affinity for the Grb2 SH2 domain was designed.[10] Molecular modeling studies suggested that the improvement in ligand binding affinity could be due to stacking between the Grb2 SH2 Arg 67 side chain and phenyl ring of mAZ and to the formation of a hydrogen bond between the mAZ amino group and the pTyr phosphate function.[11] In addition, the Cα-dialkylated residue Ac_6c is suggested to promote a favorable 3_{10} helical conformation and hydrophobic interactions of the peptide with the Grb2 SH2 domain.[10]

As mentioned above, Shc is another adaptor protein capable of connecting the EGF-R with Grb2.[12] In addition to the formerly identified Y317, another potential phosphorylation site, Y239/Y240 in the protein Shc was recently reported as an additional docking site for the Grb2 SH2 domain.[13−15] The optimal recognition motif of the Grb2 SH2 domain was constituted by a pY residue followed by a hydrophobic residue at the pY+1 position and an Asn at the pY+2 position.[16] The surrounding sequence of Shc Y239/240 is YYND. Phosphorylation of Y239 but not Y240 provides the consensus sequence pY-X-N. Furthermore, Gay et al.[17] have shown that a heptamer peptide with two adjacent phosphorylated tyrosines displayed a 25-fold higher affinity than the monophosphorylated analogue.

On the basis of all these observations, we designed in this paper a series of short phosphopeptides with a mAZ-pTyr-Xaa-Asn-NH_2 sequence, where Xaa denotes phosphotyrosine and α-methylphosphotyrosine or its carboxyl mimetics as hydrophilic residues at the pY+1 position.[18]

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Table 1

peptide	mAZ-pTyr-Xaa-Asn-NH_2	K_d (nM)[a]	IC_{50} (nM)[b]
1	mAZ-pTyr-Ac_6c-Asn-NH_2	30 ± 5	120 ± 8
2	mAZ-pTyr-pTyr-Asn-NH_2	60 ± 10	235 ± 42
3	mAZ-pTyr-(α-Me)pTyr-Asn-NH_2	3 ± 1	11 ± 1
4	mAZ-pTyr-Tyr-Asn-NH_2	155 ± 30	497 ± 50
5	mAZ-pTyr-(α-Me)Tyr-Asn-NH_2	250 ± 50	1098 ± 162
6	mAZ-pTyr-(α-Me)Phe(4-CO_2H)-Asn-NH_2	45 ± 10	153 ± 38
7	mAZ-pTyr-(α-Me)Phe(4-CH_2CO_2H)-Asn-NH_2	60 ± 10	198 ± 41

[a] Fluorescence measurements were performed on a LS250B Perkin-Elmer fluorimeter, and the Grb2−peptide equilibrium constants (K_d) were determined by the Michaelis−Menten type curve-fitting equation as described by Cussac et al.[24] [b] Competitive binding assays with recombinant Grb2 expressed as a glutathione *S*-transferase (GST) fusion protein and the immobilized phosphopeptide biotin-Aha-PSpYVNVQN were conducted using anti-GST and anti-mouse peroxidase-coupled antibodies and TMB solution. Dose−reponse relationships were constructed by nonlinear regression of the competition curves with Origin 40 software.

Chemistry

Peptides were synthesized in solid-phase peptide synthesis in Fmoc chemistry. The building block Fmoc-L-(α-Me)Tyr(PO_3Bzl_2)-OH was prepared following the general methods for preparing protected phosphotyrosine.[19] Fmoc-L-(α-Me)Phe(CO_2tBu)-OH and Fmoc-L-(α-Me)Phe(CH_2CO_2tBu)-OH were prepared following the method developed by Williams et al.,[20] using *tert*-butyl 4-(bromomethyl)benzoate[21,22] and *tert*-butyl 4-(bromomethyl)phenylacetate as alkylating agents for the enantioselective alkylation of the oxazinone derivative. These α-methylated buiding blocks and their adjacent phosphotyrosines have been coupled by the amino acid fluoride method using the coupling agent TFFH.[23] The N-terminal mAZ group was introduced as described by Furet et al.[11]

Results and Discussion

Binding Affinities. The binding affinities of these compounds for Grb2 along with that measured, in the same conditions, for the peptide **1** from Garcia-Echeverria et al.[10] are given in Table 1 as dissociation constants (K_d) measured by fluorescence.[24] The potencies of these peptides to inhibit the interaction between the Grb2 SH2 domain and phosphotyrosine-containing peptides derived from Shc Y317 are given as IC_{50} measured by an ELISA test.

Bis-phosphorylated peptide **2** shows only 2.5-fold higher binding affinity for Grb2 than its monophosphorylated analogue **4**, a lesser improvement than the one observed by Gay et al. for the heptamer peptides derived from Shc 239/240.[17] This suggests that the side-chain orientation of the second pTyr at the pY+1 position does not allow for optimal interactions with the SH2 domain. Therefore, peptide **3**, containing an α-methylated phosphotyrosine as the pY+1 residue, has been designed in order to limit the side-chain mobility upon interaction with the Grb2 SH2 domain. Moreover, α-methylation may restrict the backbone conformation of the pY+1 residue in the 3_{10} helix region of the Ramachandran plot stabilizing the type I β-turn conformation of the peptide. Indeed peptide **3** is endowed with a 20-fold higher affinity than its α-nonmethylated analogue peptide **2**. To the contrary, peptide **5** has a lower affinity than nonmethylated peptide **4**. The decreased affinity is in line with the hypothesis of a hydrophobic collapse of the steric aromatic residue at pY+1 with the N-terminal mAZ group resulting in an unfavorable preorganized conformation of the peptide in its unligated state as suggested by Garcia-Echeverria et al.[10] This collapse is probably prevented in peptide **3** by the hydrophilic nature of (α-Me)pTyr and may account for the much greater (80-fold) gain of affinity of peptide **3** to **5** versus the lesser (2.5-fold) corresponding increased affinity of **2** relative to **4**. Moreover, peptide **3** has a 10-fold higher affinity than the molecule designed by Garcia-Echeverria et al. (peptide **1**).[10] Thus, to evaluate the importance of the negative charges of the phosphate of the (α-Me)pTyr residue, two α-methylated L-phenylalanine-containing derivatives bearing a carboxyl group in the *para* position were synthesized and incorporated as pY+1 in the peptide sequence (compounds **6** and **7**). The affinities of both peptides are about 15−20-fold lower than that of peptide **3** but in the range of those of **1** and **2**. The pY+1 residue probably needs a highly negatively charged group in order to fulfill the network of its hydrogen bond interactions with the residues of the Grb2 SH2 domain.

Finally, confirming these results, Table 1 also shows that in an ELISA test, peptide **3**, endowed with the highest affinity for Grb2, is also able to inhibit at a very low dose (IC_{50} = 11 ± 1 nM) the interaction between Grb2 and a phosphopeptide derived from Shc Y317.

Molecular Modeling. Molecular modeling of peptide **3** docked within the Grb2 SH2 domain (Figure 1) shows that the complex is stabilized by numerous ionic hydrogen bonds and stacking interactions. The pTyr and Asn residues bind in a manner similar to that observed in the crystal structure of the complex of the phosphopeptide KPFpYVNV with the Grb2 SH2 domain.[9] As observed by Furet et al.,[11] in the case of mAZ-pTyr-Ac_6c-Asn-NH_2, the mAZ group contributes additional interactions by a hydrogen bonding between its carbonyl oxygen and the guanidiniun group of Arg 67, which is also superimposed on the phenyl ring of mAZ. The phosphate group of the (α-Me)pTyr residue forms hydrogen bonds with the side chains of Arg 142, Asn 143, and Ser 141 and the backbone amide groups of Arg 142 and Asn 143. In addition, the aromatic ring of (α-Me)-pTyr is stacked on the side chain of Phe 108. The onset of these multiple hydrogen-bonding interactions can explain the decrease of affinities of peptides **6** and **7**, as these compounds have a single negative charge on the carboxylate instead of the double charge on the phosphate group of **3**.

Conclusion

In conclusion, our results demonstrate that a hydrophobic residue at pY+1 can be advantageously replaced by a constrained (α-Me)pTyr residue, which can provide

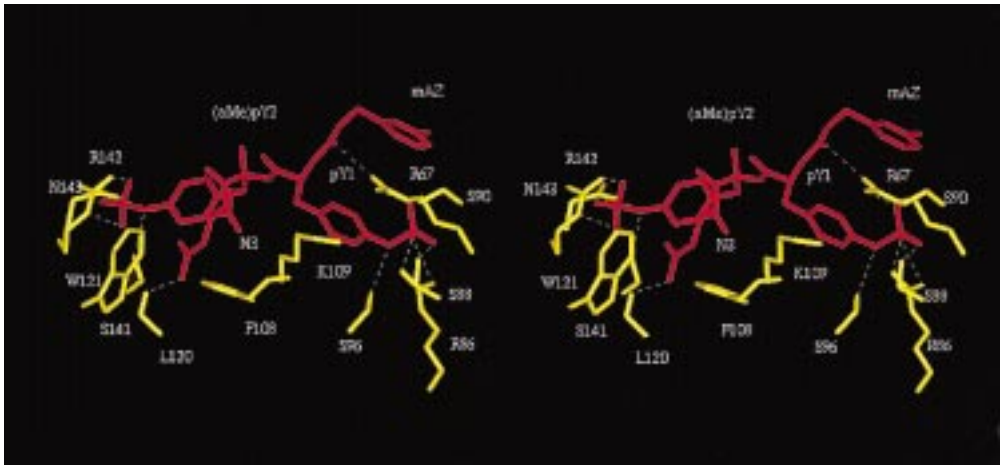

Figure 1. Stereoview representation of the most important interactions occurring between peptide **3** and the binding site of the Grb2 SH2 domain, as derived from molecular dynamics calculations performed with the MSI[26] software and the AMBER force field.

both hydrophobic and additional hydrogen bond interactions. Among the small phosphotripeptides designed to date, compound **3** appears to have the highest binding affinity for Grb2 as measured directly.[10,25] The crystallization of this peptide with the Grb2 SH2 domain is now in progress and should provide additional insights for the rational design of nonpeptide inhibitors of Grb2.

Experimental Section

Rink MBHA amide resin was purchased from NovaBiochem. TFFH was from Perseptive Biosystems and Fmoc-Tyr(PO_3-$MDPSE_2$)-OH from Bachem Inc. The other reagents for solid-phase peptide synthesis were from Applied Biosystems and the reagents for chemical preparations from Aldrich. The NMR spectra were recorded on a Bruker WH270 spectrometer operating at 270 MHz or at 400 MHz in the case of peptides. Chemical shifts are given in ppm relative to HMDS as internal standard.

Fmoc-L-(α-Me)Tyr-OH. To a solution of L-(α-Me)Tyr-OH (500 mg, 2.56 mmol) dissolved in 0.5 N NaOH (10.4 mL, 5.2 mmol) was added a solution of Fmoc-Cl (1.35 g, 5.2 mmol) in acetonitrile (5.2 mL). The resulting mixture was stirred at room temperature for 3 h, and acetonitrile was then evaporated. Aqueous residue was added to 10% Na_2CO_3 (25 mL), extracted with ether, acidified with 6 N HCl, and extracted with ethyl acetate. The EtOAc extract was washed successively with 2 N HCl, H_2O, and brine and dried over Na_2SO_4. The residue obtained after evaporation of solvent was purified by column chromatography on silica gel (eluted with CH_2Cl_2/MeOH/AcOH, 95/5/1) to give 1.09 g of Fmoc-L-(α-Me)Tyr-OH as a white powder (yield: 100%). R_f = 0.58 (CH_2Cl_2/MeOH/AcOH, 95/5/5). ^{1}H NMR (DMSO-d_6): 1.15 (s, 3H, α-Me), 2.75, 3.05 (dd, 2H, $CH_2\beta$), 4.25 (m, 2H, 9′-CH_2 of Fmoc), 4.45 (m, 1H, 9′-H of Fmoc), 6.55, 6.75 (dd, 4H, H−Ar of Tyr), 7.15 (s, 1H, NH), 7.30 (t, 2H, 2′,7′-H of Fmoc), 7.40 (t, 2H, 3′,6′-H of Fmoc), 7.70 (d, 2H, 4′,5′-H of Fmoc), 7.85 (d, 2H, 1′,8′-H of Fmoc), 9.12 (s, 1H, OH).

Fmoc-L-(α-Me)Tyr(PO_3Bzl_2)-OH. This compound was prepared following the method described for the preparation of Fmoc-Tyr(PO_3Bzl_2)-OH,[19] with Fmoc-L-(α-Me)Tyr as starting material. Fmoc-L-(α-Me)Tyr(PO_3Bzl_2)-OH was obtained as a white powder with a yield of 84%. R_f = 0.40 (CH_2Cl_2/MeOH, 95/5). ^{1}H NMR (DMSO-d_6): 1.25 (s, 3H, α-Me), 3.02 (m, 2H, $CH_2\beta$), 4.15 (m, 2H, 9′-CH_2 of Fmoc), 4.30 (m, 1H, 9′-H of Fmoc), 5.07 (d, 4H, CH_2 of Bzl), 6.85, 6.95 (dd, 4H, H−Ar of Tyr), 7.3 (m, 15H, NH, H−Ar of Bzl and 2′,3′,6′,7′-H of Fmoc), 7.50, 7.55 (dd, 2H, 4′,5′-H of Fmoc), 7.80 (m, 2H, 1′,8′-H of Fmoc).

***tert*-Butyl 4-(Bromomethyl)benzoate.** *tert*-Butyl 4-methylbenzoate[21] (12.00 g, 62.4 mmol) was brominated with *N*-bromosuccinimide (12.00 g, 67.4 mmol), initiated by dibenzoyl peroxide (0.82 g, 3.4 mmol) in CCl_4, following the procedure that we have described.[22] The crude product obtained was purified by column chromatography on silica gel (eluted with AcOEt/c-hexane, 1/15) to give 13.0 g of *tert*-butyl 4-(bromomethyl)benzoate as a colorless oil (yield: 77%). R_f = 0.42 (AcOEt/c-hexane, 1/15). ^{1}H NMR (DMSO-d_6): 1.47 (s, 9H, tBu), 4.65 (s, 2H, CH_2Br), 7.50 and 7.85 (dd, 4H, H−Ar).

(3*S*,5*S*,6*R*)-4-(Benzyloxycarbonyl)-5,6-diphenyl-3-methyl-3-[4′-(*tert*-butyloxycarbonyl)benzyl]-2,3,5,6-tetrahydro-4*H*-1,4-oxazin-2-one (8). To a solution of (3*S*,5*S*,6*R*)-4-(benzyloxycarbonyl)-5,6-diphenyl-3-methyl-2,3,5,6-tetrahydro-4*H*-1,4-oxazin-2-one[20] (1.00 g, 2.5 mmol) in anhydrous THF (20 mL), at −78 °C, was added dropwise under nitrogen a 0.5 M solution KHMDS in toluene (15 mL, 7.5 mmol). The resulting solution was stirred for 15 min; then a solution of *tert*-butyl 4-(bromomethyl)benzoate (4.0 g, 14.7 mmol) dissolved in THF (10 mL) was added dropwise. The mixture was stirred at −78 °C for 0.5 h and then pourred into AcOEt (100 mL). The organic solution was washed with H_2O (1 × 25 mL) and brine (1 × 25 mL) and dried over Na_2SO_4. After filtration and evaporation of solvent, the residue was purified by column chromatography on silica gel (eluted with AcOEt/c-hexane, 1/10) to give 650 mg of product **8** as a white powder (yield: 44%). R_f = 0.32 (AcOEt/c-hexane, 1/10). ^{1}H NMR (DMSO-d_6): 1.50 (s, 9H, tBu), 1.80 (s, 3H, 3-Me), 3.25 and 3.95 (dm, 2H, CH_2), 4.55 (s, 1H, 5-H), 5.55 (s, 2H, CH_2 of Cbz), 5.70 (d, 1H, 6-H), 6.70 (m, 4H, H−Ar), 7.0−7.3 (m, 13H, NH and H−Ar), 7.80 (d, 2H, H−Ar).

L-(α-Me)Phe(4-CO_2tBu)-OH (9). To a solution of compound **8** (60 mg, 0.10 mmol) in THF/EtOH (1/1, 4 mL) was added 10% Pd−C (6 mg), and the suspension was hydrogenated overnight. The catalyst was then filtered off and the filtrate concentrated and precipitated with ether. The precipitate was centrifuged to give 28 mg of product **9** as a white powder (yield: 99%). ^{1}H NMR (DMSO-d_6 + TFA): 1.40 (s, 3H, α-Me), 1.48 (s, 9H, tBu), 3.05 (q, 2H, CH_2), 7.25 et 7.80 (dd, 4H, H−Ar), 8.25 (s, 3H, NH_3^+).

Fmoc-L-(α-Me)Phe(4-CO_2tBu)-OH (10). To a solution of compound **9** (188 mg, 0.673 mmol) in dioxane/10% $NaHCO_3$ (1/1, 30 mL) was added Fmoc-Cl (500 mg, 1.93 mmol). The resulting mixture was stirred at room temperature for 3 h, and solvent was then evaporated. Aqueous residue was added to 5% $NaHCO_3$ (25 mL), washed with ether, acidified with 10% citric acid to pH 2, and extracted with EtOAc. The EtOAc extract was washed successively with 10% citric acid, H_2O, and brine and dried over Na_2SO_4. The residue obtained after solvent evaporation was purified by column chromatography on silica gel (eluted with CH_2Cl_2/MeOH, 95/5) to give 280 mg of product **10** as a white powder (yield: 83%). R_f = 0.18 (CH_2Cl_2/MeOH, 95/5). ^{1}H NMR (DMSO-d_6): 1.15 (s, 3H, α-Me), 1.50

(s, 9H, tBu), 2.95 and 3.25 (dd, 2H, CH_2), 4.20 (t, 1H, 9′-H of Fmoc), 4.25 and 4.40 (mm, 2H, 9′-CH_2 of Fmoc), 7.05 (3H, NH and 2,6-H−Ar of Phe), 7.28 (t, 2H, 2′,7′-H of Fmoc), 7.35 (t, 2H, 3′,6′-H of Fmoc), 7.65 (m, 4H, 3,5-H−Ar of Phe and 4′,5′-H of Fmoc), 7.85 (d, 2H, 1′,8′-H of Fmoc).

***tert*-Butyl 4-(Bromomethyl)phenylacetate.** A solution of 4-(bromomethyl)phenylacetic acid (9.7 g, 42.34 mmol), in thionyl chloride (100 mL), was refluxed for 3 h and then evaporated to dryness. The solid residue was dissolved in a minimal volume of CH_2Cl_2 (4 mL) and added dropwise to a solution of *tert*-butyl alcohol (140 mL) and CH_2Cl_2 (5 mL) cooled at 0 °C. The resulting solution was stirred at 4 °C overnight and then added to CH_2Cl_2 (100 mL). The organic phase was washed successively with H_2O, 10% $NaHCO_3$, and H_2O and dried over Na_2SO_4. Evaporation of the solvent to dryness gave 10.9 g of product as a light yellow solid (yield: 91%). $R_f = 0.70$ (CH_2Cl_2). ^{1}H NMR (DMSO-d_6): 1.35 (s, 9H, tBu), 3.50 (s, 2H, CH_2CO_2), 4.65 (s, 2H, CH_2Br), 7.20, 7.35 (dd, 4H, H−Ar).

(3*S*,5*S*,6*R*)-4-(Benzyloxycarbonyl)-5,6-diphenyl-3-methyl-3-[4′-((*tert*-butyloxycarbonyl)methyl)benzyl]-2,3,5,6-tetrahydro-4*H*-1,4-oxazin-2-one (11). Compound **11** was prepared following the method described for preparing compound **8**, using the *tert*-butyl 4-(bromomethyl)phenylacetate as alkylating agent (yield: 31%). $R_f = 0.25$ (EtOAc/c-hexane, 1/10). ^{1}H NMR (DMSO-d_6): 1.30 (s, 9H, tBu), 1.85 (s, 3H, 3-Me), 3.1 and 4.0 (dm, 2H, $CH_2\beta$), 3.55 (s, 2H, CH_2CO_2), 4.25 (s, 1H, 5-H), 5.1 (m, 3H, CH_2 of Cbz and 6-H), 6.65−7.30 (m, 20H, NH and H−Ar).

L-(α-Me)Phe(4-CH_2CO_2tBu)-OH (12). Compound **12** was prepared following the method described for preparing compound **9** (yield: 90%). ^{1}H NMR (DMSO-d_6): 1.30 (s, 3H, α-Me), 1.35 (s, 9H, tBu), 3.0 (q, 2H, $CH_2\beta$), 3.50 (s, 2H, CH_2CO_2), 7.15 (m, 6H, NH_2 and H−Ar).

Fmoc-L-(α-Me)Phe(4-CH_2CO_2tBu)-OH (13). Compound **13** was prepared following the method described for preparing compound **10** (yield: 50%). $R_f = 0.12$ (CH_2Cl_2/MeOH, 95/5). ^{1}H NMR (DMSO-d_6): 1.20 (s, 3H, α-Me), 1.35 (s, 9H, tBu), 3.0 (q, 2H, $CH_2\beta$), 3.40 (s, 2H CH_2CO_2), 4.15 (t, 1H, 9′-H of Fmoc), 4.28 (m, 2H, 9′-CH_2 of Fmoc), 6.95 (q, 4H, H−Ar of Phe), 7.26 (t, 2H, 2′,7′-H of Fmoc), 7.35 (t, 2H, 3′,6′-H of Fmoc), 7.6 (m, 3H, NH and 4′,5′-H of Fmoc), 7.85 (d, 2H, 1′,8′-H of Fmoc).

Peptide Synthesis. Peptide synthesis was performed on an Applied Biosystems (ABI) 431A peptide synthesizer with ABI small-scale Fmoc chemistry. Fmoc-Asn(Trt)-OH (1 mmol) was coupled by DCC/HOBt to Fmoc pre-deprotected Rink MBHA amide resin (200 mg, 0.1 mmol), and the Fmoc group of Asn was then removed by 20% of piperidine. The α-methylated Fmoc-protected amino acid (0.5 mmol) was activated for less than 5 min with TFFH (0.5 mmol) and DIEA (1.0 mmol) in DMF (4 mL),[23] the resulting solution was transferred to the peptidyl resin, and the coupling was carried out for 4 h. The α-nonmethylated amino acid was introduced by the BOP/HOBt/DIEA coupling method. The following Fmoc-Tyr(PO_3-$MDPSE_2$)-OH was coupled either by TFFH/DIEA to α-methylated amino acid residue or by BOP/HOBt/DIEA to α-nonmethylated residue on the resin. After deprotection of the Fmoc group, Boc-mAZ-ONp (1 mmol)[11] was coupled in the presence of DIEA (1.2 mmol) overnight. The final peptidyl resin was then dried and cleaved with a mixture of TFA/TIPS/H_2O (9.5/0.25/0.25 in volume) for 3 h at room temperature. The filtrate from the cleavage reaction was precipitated with cold ether, and the precipitate was collected by centrifugation. The crude peptide was purified by semipreparative HPLC on a Nucleosil C_{18} column (Vydac, 5 μm, 10 × 250 mm), and the fractions were analyzed by analytical HPLC on a Nucleosil C_{18} column (Vydac, 5 μm, 4.6 × 150 mm). The pure fractions were collected and lyophilized. The structure of the peptides was confirmed by electrospray mass and NMR spectroscopy.

Abbreviations: Ac_6c, 1-aminocyclohexanecarboxylic acid; Aha, 6-aminohexanoic acid; Asn or N, asparagine; Boc, *tert*-butyloxycarbonyl; BOP, (1*H*-benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; DIEA, diisopropylethylamine; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; mAZ, 3-aminobenzyloxycarbonyl; MDPSE, (methyldiphenylsilyl)ethyl; Phe or F, phenylalanine; pTyr or pY, phosphotyrosine; TFA, trifluoroacetic acid; TFFH, tetramethylfluoroformamidinium hexafluorophosphate; TIPS, triisopropylsilane; Trt, trityl; Tyr or Y, tyrosine.

Molecular Modeling. Molecular dynamics calculations were done with the MSI[26] software and the AMBER force field. A distance-dependent dielectric screening of 4*r* was used. The protein was built out from residues 55−152, using as a starting point the PDB crystal structure of the SH2 domain complexed with ligand KPFpYVNV (PDB entry 1PTZ).[9] Manual docking and preliminary rounds of constrained energy minimizations were first performed using our computer graphics facilities in order to prevent steric clashes between the (α-Me)pTyr ring and Trp 121, while favoring the attractive interaction with Arg 142. For molecular dynamics, the protein backbone was held frozen but its side chains are relaxed. The ligand was completely relaxed. After 5000-fs initialization steps at 300 K, 100 steps of molecular dynamics calculations were performed. Each was done at 300 K during 5000 steps of 1 fs. The resulting structure was submitted to conjugate-gradient energy minimization and stored. All 100 structures are characterized by ionic interactions between each of the pTyr residues and neighboring arginines. They present strong mutual overlaps and close total energies.

Affinity Measurement. Fluorescence measurements were performed on a LS250B Perkin-Elmer fluorimeter in a 10- × 10-mm cuvette at 25 °C, as described by Cussac et al.[24] Briefly, the excitation was at 292 nm (bandwidth 5.0 nm), and emission was recorded at 345 nm (bandwidth 5.0 nm). The buffer was Hepes (50 mM, pH 7.5), DTT (1 mM). The constants K_d were determined by the Michaelis−Menten type curve-fitting equation.[24]

Competition Assay. Precoated streptavidin plates (Boehringer) were incubated with 100 μL/well of biotin-Aha-PSpYVNVQN peptide (100 nM concentration in PBS buffer) overnight at 4 °C. Nonspecific binding was blocked with PBS/3% BSA during 4 h at 4 °C. Competitors were incubated, at the appropriate concentrations, in PBS/3% milk containing 40 nM GST-Grb2 protein (100 μL/well) during one night at 4 °C. Revelation is made after anti-GST (Transduction Laboratories; 1/500 in PBS/milk/0.05% Tween 20) and peroxidase-coupled anti-mouse (Amersham; 1/1000 in PBS/milk/0.05% Tween 20) incubations, using TMB solution (Interchim). After coloration was stopped with H_2SO_4 (10% v/v), OD was read at 550 nm. Dose−response relationships were constructed by nonlinear regression of the competition curves with Origin 40 software.

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Supporting Information Available: Analytical data (NMR and MS) for peptides **2**−**7** is available free of charge via the Internet at http://pubs.acs.org.

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(25) During preparation of this article, a paper from Schoepfer et al. (Schoepfer, J.; Fretz, H.; Gay, B.; Furet, P.; Garcia-Echeverria, C.; End, N.; Caravatti, G. Highly potent inhibitors of the Grb2-SH2 domain. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 221−226) was published. It reported that a 3-indolylpropylamine phosphotripeptide was able to inhibit, in an ELISA test, the binding of the phosphorylated carboxy-terminal intracellular domain of EGF-R to Grb2 SH2 with an IC_{50} of 0.3 nM. This represents a 3-fold improvement with respect to compound **1**, whose actual affinity for Grb2 was measured by fluorescence in our paper as a K_d value of 30 nM, while peptide **3** has a 10-fold better K_d of 3 nM.
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