Pergamon

Bioorganic & Medicinal Chemistry, Vol. 3, No. 9, pp. 1263–1272, 1995

0968-0896/95 $9.50 + .00

0968-0896(95)00112-3

Structure–Activity Studies of Phosphorylated Peptide Inhibitors of the Association of Phosphatidylinositol 3-Kinase with PDGF-β Receptor

Kalaiyarasi Ramalingam,[a] Scott R. Eaton,[a] Wayne L. Cody,[a]* Gina H. Lu,[b] Robert L. Panek,[b] Lisa A. Waite,[a] Stuart J. Decker,[c] Joan A. Keiser[b] and Annette M. Doherty[a]

Departments of [a]Chemistry, [b]Cardiovascular Therapeutics and [c]Signal Transduction, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, MI 48105, U.S.A.

Abstract—Phosphorylated pentapeptides derived from Tyr^{751} of the PDGF-β receptor ($pTyr^{751}$-Val-Pro-Met-Leu, pTyr = phosphotyrosine) were prepared to examine their ability to inhibit the association of the C-terminal SH2 domain of the p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase) with the PDGF-β receptor. Peptidic analogs were prepared to examine the importance of the amine and carboxy terminus and specific amino acids via alanine/D-amino acid scans and site specific modifications. Several of these peptides had submicromolar activity. In particular, it was shown that neutralization of the amine and carboxy terminus led to analogs with enhanced activity. In addition, it was determined that only minimal modifications were allowed for pTyr and Met, while the other positions were quite tolerant of modification.

Introduction

The proliferation, differentiation and survival of cells are regulated by numerous extracellular signaling polypeptides. Thus, these signaling polypeptides better known as growth factors have been implicated in several disease states that involve uncontrolled cellular proliferation and differentiation such as cancer, atherosclerosis, and restenosis. Some of the better characterized growth factors include the following: epidermal growth factor (EGF), fibroblast growth factors (FGFs), and platelet derived growth factor (PDGF).[1–3]

The FGFs and PDGF have been shown to be potent vascular smooth muscle cell (VSMC) mitogens and chemoattractants.[4,5] The proliferation and directed migration of VSMCs have been identified as critical factors in the onset of restenosis. In response to balloon-injury of rat carotid arteries an upregulation of PDGF and PDGF receptor isoforms has been reported.[6] In addition, in this model of restenosis the infusion of PDGF was shown to greatly increase neointimal thickening and VSMC migration.[7] However, antibodies raised to PDGF were shown to significantly inhibit VSMC migration and neointimal thickening following balloon injury.[8] Clinically, 20–45% of patients that undergo percutaneous transluminal coronary angioplasty (PTCA) suffer from restenosis within 3–6 months of the procedure.[9,10]

The binding of growth factors to extracellular receptors activates intracellular tyrosine kinases that catalyze the phosphorylation of several tyrosines on intracellular protein substrates or the receptor (autophosphorylation). These phosphorylated tyrosines create high affinity binding sites for many secondary cellular proteins involved in signal transduction such as phosphatidylinositol 3-kinase (PI 3-kinase), phospholipase C-γ (PLC-γ) and ras-GTPase-activating protein (GAP).[1–3] These molecules contain homologous regions known as src homology 2 (SH2) domains that were first identified in src family protein tyrosine kinases (PTKs).[11,12] SH2 domains confer high affinity inter-actions with specific phosphorylated tyrosine (pTyr or pY) residues of the growth factor receptors.[13] Further downstream signaling results in cellular proliferation. Thus, the blockade of these signal transduction pathways has significant implications in the treatment of proliferative diseases.

In particular, the binding of PDGF to cell surface receptors induces receptor dimerization, followed by autophosphorylation at multiple tyrosine residues which initiate the cytoplasmic signaling via secondary cellular proteins containing SH2 domains.[14–16] Currently, more than twenty cytosolic proteins likely to be involved in signaling have been shown to contain SH2 domains. Of these, PI 3-kinase is an important member which interacts with many activated PTKs and is involved in both normal and oncogenic signal transduction.[17,18] The role of PI 3-kinase in PDGF-mediated cell proliferation has been suggested by measuring the levels of DNA synthesis in NMuMG cells expressing wild-type versus those expressing mutant PDGF receptors. A significant increase in DNA synthesis was observed in cells expressing PDGF receptors that were specifically associated with PI 3-kinase.[19]

PI 3-kinase is a heterodimeric enzyme and contains an 85 kDa (p85) non-catalytic subunit and a 110 kDa (p110) catalytic subunit.[20,21] The p85 subunit has one src homology 3 (SH3) and two SH2 domains which bind to specific phosphorylated tyrosines on activated growth factor receptors. PI 3-kinase has been shown to interact specifically with the phosphorylated Tyr^{740} and Tyr^{751} residues of the PDGF-β receptor.[22] These sites on the PDGF-β receptor were identified by using small (5–6 amino acids) synthetic phosphopeptides as competitive inhibitors to inhibit the association of the PI 3-kinase to PDGF-β receptor.[19] In particular, two peptides ($pTyr^{740}$-Met-Asp-Met-Ser and $pTyr^{751}$-Val-Pro-Met-Leu) from the PDGF-β receptor sequence provided 90% inhibition of the association at 100 μM concentration. In particular, it was shown that pTyr and Met at the pY + 3 position (Fig. 1) were crucial for binding to PI 3-kinase. Synthetic phosphotyrosine containing peptides with Gly, Ala or Pro substituted in the pY + 3 position did not bind.[19]

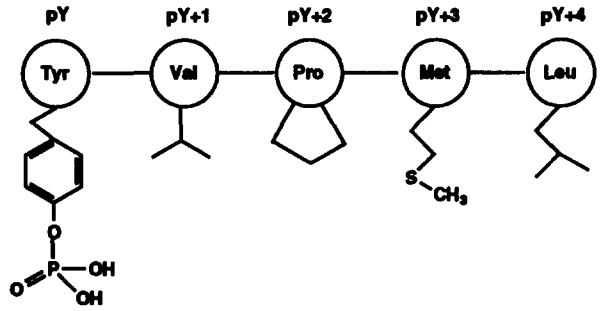

Figure 1. Structure of peptide **1** derived from the Tyr^{751} region of the PDGF-β receptor. The amino acids are labeled as pY + 1, + 2, + 3 and + 4 C-terminal to the pTyr (pY) residue.

Specific amino acid sequences that recognize the SH2 domains of several intracellular proteins including PI 3-kinase have been identified using synthetic peptides or phosphopeptide library approaches.[19,23] These motifs include the following: pTyr-Met-Met-Met-Arg (Tyr^{766} of the FGF receptor), pTyr-Thr-His-Met-Asn (Tyr^{1334} of the insulin receptor), pTyr-Glu-Val-Met-Leu (Tyr^{1331} of the hepatocyte growth factor receptor), pTyr-Met-Asp-Met-Lys (Tyr^{721} of the steel receptor), and pTyr-Val-Glu-Met-Arg (Tyr^{723} of the CSF-1 receptor).[19] A peptide library was used for the determination of these optimal sequences based on the degenerate phosphorylated sequence of Gly-Asp-Gly-pTyr-Xxx-Xxx-Xxx-Ser-Pro-Leu-Leu-Leu, where Xxx was a mixture of the 20 naturally occurring amino acids.[23] The optimal sequences discovered for binding to the PI 3-kinase p85 SH2 domains (pTyr-Val-Xxx-Met or pTyr-Met-Xxx-Met) were the same sequences previously identified for PDGF[19] and polyomavirus middle T antigen.[24] In addition, a protein–tyrosine substrate library identified the sequence of Tyr-hydrophobic-Xxx-hydrophobic as the preferred substrate for PDGF receptor tyrosine kinase.[25]

Of the two SH2 domains (N- and C-terminal) of the p85 subunit of PI 3-kinase, the C-terminal SH2 domain like the full-length p85 distinguishes between the wild-type and a mutant PDGF receptor lacking the PI 3-kinase binding site. Thus, the C-terminal SH2 domain of the p85 subunit (p85 C-SH2) accounts for the high affinity and specificity of the binding of PI 3-kinase to the PDGF-β receptor.[13] Therefore, we have concentrated our study on inhibiting of the association of the p85 C-SH2 of PI 3-kinase and the intracellular tyrosine kinase domain of the PDGF-β receptor (PDGFR-TK).

The goal of our study was to delineate the structural determinants of the high affinity binding of phosphopeptides to p85 C-SH2 of PI 3-kinase using a series of synthetic peptide analogs based upon the phosphopentapeptide sequence pTyr-Val-Pro-Met-Leu (derived from Tyr^{751} of the PDGF-β receptor). In this report, we will describe the structure–activity relationships of this phosphopentapeptide sequence determined from the following: (a) N- and C-terminal modifications (Table 1); (b) an alanine scan (Table 2); (c) a D-amino acid scan (Table 2); (d) individual amino acid substitutions (Table 3); and C-terminal truncations (Table 4).

Results and Discussion

Previously, Fantl *et al.*[19] had prepared a series of pentapeptides corresponding to Tyr^{740} through Tyr^{751} of the PDGF-β receptor and examined their ability to block the association of PI 3-kinase with the PDGF receptor. This study identified the amino acid residues at the pY and pY + 3 position in the phosphopentapeptide segment, pTyr-Xxx-Xxx-Met-Xxx as being crucial for binding. Specifically, it was shown that the synthetic pentapeptide sequences, $pTyr^{751}$-Val-Pro-Met-Leu (**1**), pTyr-Val-Ala-Met-Leu (**6**) and $pTyr^{740}$-Met-Asp-Met-Ser were able to block 90% of the association of PDGF-β receptor with p85 C-SH2 at 100 μM.[19] We

Table 1. Effect of N- and C-terminal modifications of peptide **1** for inhibiting the interaction of p85 C-SH2 with PDGFR-TK

Peptide	Sequence	IC_{50} (μM)	N.D.*
1	Tyr(PO_3H_2) - Val - Pro - Met - Leu	0.67	33
N- and C-Terminal Modifications:			
2	Ac - Tyr(PO_3H_2) - Val - Pro - Met - Leu	0.18	2
3	Tyr(PO_3H_2) - Val - Pro - Met - Leu - NH_2	0.40	2
4	Ac - Tyr(PO_3H_2) - Val - Pro - Met - Leu - NH_2	0.16	2

*Number of determinations.

Table 2. Effect of alanine and D-amino acid substitutions of peptide 1 for inhibiting the interaction of p85 C-SH2 with PDGFR-TK

Peptide	Sequence	IC_{50} (μM)	N.D.*
1	pTyr - Val - Pro - Met - Leu	0.67	33
Alanine Scan:			
5	pTyr - **Ala** - Pro - Met - Leu	8.4	2
6	pTyr - Val - **Ala** - Met - Leu	2.7	2
7	pTyr - Val - Pro - **Ala** - Leu	†	2
8	pTyr - Val - Pro - Met - **Ala**	0.34	2
D-Amino Acid Scan:‡			
9	**ptyr** - Val - Pro - Met - Leu	†	2
10	pTyr - **val** - Pro - Met - Leu	†	2
11	pTyr - Val - **pro** - Met - Leu	†	2
12	pTyr - Val - Pro - **met** - Leu	†	1
13	pTyr - Val - Pro - Met - **leu**	5.7	4

*Number of determinations.
†Inactive (less than 50% inhibition at 50 μM peptide concentration).
‡Lower case indicates a D-amino acid.

Table 3. Effect of individual modifications of peptide 1 for inhibiting the interaction of p85 C-SH2 with PDGFR-TK

Peptide	Sequence				IC_{50}(μM)	N.D.*
	pY					
1	**pTyr**	- Val	- Pro	- Met - Leu	0.67	33
14	**Tyr**	- Val	- Pro	- Met - Leu	†	2
15	**pSer**	- Val	- Pro	- Met - Leu	†	2
16	**pThr**	- Val	- Pro	- Met - Leu	†	2
17	**pHO-Cha‡**	- Val	- Pro	- Met - Leu	†	2
18	**pHO-Tic‡**	- Val	- Pro	- Met - Leu	†	1
		pY + 1				
19	pTyr	- **Nle**	- Pro	- Met - Leu	2.0	2
20	pTyr	- **Chg‡**	- Pro	- Met - Leu	7.2	2
21	pTyr	- **Asp**	- Pro	- Met - Leu	3.5	2
22	pTyr	- **Glu**	- Pro	- Met - Leu	6.0	3
			pY + 2			
23	pTyr	- Val	- **Azt‡**	- Met - Leu	1.7	2
24	pTyr	- Val	- **Pip‡**	- Met - Leu	6.7	2
25	pTyr	- Val	- **Inp‡**	- Met - Leu	†	3
26	pTyr	- Val	- **Oic‡**	- Met - Leu	3.8	2
27	pTyr	- Val	- **Aib‡**	- Met - Leu	11.1	2
28	pTyr	- Val	- **MeA**	- Met - Leu	3.9	1
				pY + 3		
29	pTyr	- Val	- Pro	- **Met(O)** - Leu	†	2
30	pTyr	- Val	- Pro	- **$Met(O)_2$** - Leu	†	2
31	pTyr	- Val	- Pro	- **Met(SBzl)*‡** - Leu	†	1
32	pTyr	- Val	- Pro	- **Dab‡** - Leu	†	1
33	pTyr	- Val	- Pro	- **Ser** - Leu	†	2
34	pTyr	- Val	- Pro	- **Cys(SMe)** - Leu	12.6	2
35	pTyr	- Val	- Pro	- **Nle** - Leu	1.4	19

*Number of determinations.
†Inactive (less than 50% inhibition at 50 μM peptide concentration).
‡Structures of the unusual amino acids are shown in Figure 2.

Table 4. Effect of C-terminal truncations of peptide 1 for inhibiting the interaction of p85 C-SH2 with PDGFR-TK

Peptide	Sequence	IC_{50} (μM)	N.D.*
1	$Tyr(PO_3H_2)$ - Val - Pro - Met - Leu	0.67	33
N- and C-Terminal Modifications:			
36	$Tyr(PO_3H_2)$ - Val - Pro - Met	10.1	2
37	$Tyr(PO_3H_2)$ - Val - Pro - Met - NH_2	1.3	2
38	Ac - $Tyr(PO_3H_2)$ - Val - Pro - Met - NH_2	0.25	18

*Number of determinations.

have been able to quantitatively determine the ability of peptides **1** and **6** to block this association [IC_{50} = 0.67 (Table 1) and 2.7 μM (Table 2), respectively]. [pTyr[740]-Met-Asp-Met-Ser had an IC_{50} of 0.52 μM (n = 2) in our hands, but is not the subject of this report.]

In addition, Songyang *et al.*,[23] used a degenerate phosphorylated tyrosine peptide library approach to determine the optimal sequence for blocking the association to the N- and C-terminal SH2 domains of PI 3-kinase. In particular, this study showed that hydrophobic residues were preferred at the pY + 1 position and Met was preferred at the pY + 3 position. For the C-terminal SH2 domain, the amino acid motif that was selected was pTyr-hydrophobic-Xxx-Met.[23] Therefore, we decided to utilize the phosphopentapeptide pTyr[751]-Val-Pro-Met-Leu (Fig. 1, peptide **1**) as a template for our structure–activity study.

N- and C-terminal modifications (Table 1)

The role of the charged amino and carboxy terminus was examined by N-terminal acetylation and/or C-terminal amidation of the parent peptide (**1**). Either modification enhanced the ability of the phosphorylated pentapeptide to block the association of PDGFR-TK with the p85 C-SH2. In particular, the N-terminal acetylated and C-terminal amidated analog (peptide **4**) had significantly improved affinity (IC_{50} = 0.16 μM).

This increase in binding affinity to p85 C-SH2 for the acetylated and amidated peptides (**2–4**) over that of peptide (**1**) may be attributed to neutralization of the charges at either terminus. In the crystal structures[26–29] and solution NMR-derived structure (H_2O, pH 5.5)[30] of src SH2 domain complexes with phosphorylated peptide substrates the phosphate of pTyr is located in a well defined pocket which seems to be conserved in most SH2 domain containing proteins. In these complexes, the pY-1 peptide carbonyl is involved in hydrogen bond interactions with the SH2 domain residues. Such hydrogen bond interactions may also be formed by peptides (**2** and **4**) as a result of the acetylation of their N-termini thus increasing the binding affinity. The enhanced binding affinity of peptide **3** may simply be a result of diminished charge–charge interactions or enhanced hydrophobic interactions with p85 C-SH2.

Alanine scan (Table 2)

An alanine scan was performed on peptide **1**. The substitution of alanine for the naturally occurring residue eliminates the contribution of the side-chain while maintaining the configuration of the peptide backbone (Table 2). Therefore, the difference in potency between the alanine-substituted and the naturally occurring peptide provides an understanding of the contribution of the side-chain functionality to binding.[31] Peptides **5** and **6** with alanine at the pY + 1 and pY + 2 positions showed moderate binding affinity. However, the substitution of Ala for Met at the pY + 3 position (peptide **7**) completely abolished the binding affinity reiterating the importance of this side chain.[15] Substitution of Ala at the pY + 4 position (peptide **8**) exhibited similar activity to peptide **1**.

D-Amino acid scan (Table 2)

The substitution of D-amino acids for L-amino acids in peptides helps to define the importance of the three-dimensional orientation of the side chain to molecular recognition.[32] Each amino acid in peptide **1** was replaced with its corresponding D-isomer (Table 2). Except for the substitution of a D-amino acid at pY + 4, all of the substitutions led to inactive analogs. Although this type of substitution should not substantially effect the overall physicochemical properties of the molecule, it may have a profound effect on the local conformation of the peptide backbone. The large decrease in the activity of the D-amino acid substituted peptides at the pY + 1 to pY + 3 positions suggests the importance of the side chain orientation of these amino acids in peptide **1** for interaction with p85 C-SH2. Since the substitution of a D-amino acid in the pY + 4 position did not lead to an inactive analog, this suggests that this position is not as critical for this interaction.

Substitutions at the pY position (Table 3).

The interaction of the SH2 domains of the p85 subunit of PI 3-kinase with the activated growth factor receptors is specific for pTyr residues. We have further examined the importance of the pTyr for the association of PDGFR-TK with the p85 C-SH2 by synthetic peptides in which the pTyr residue was replaced by the following amino acids: Tyr (**14**); phosphoserine (pSer, **15**); phosphothreonine (pThr, **16**); phospho-4-hydroxy-cyclohexylalanine (pHO-Cha, **17**); phospho-7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (pHO-Tic, **18**) (Table 3 and Fig. 2). The unphosphorylated Tyr containing peptide (**14**) did not bind to p85 C-SH2 and further emphasizes the importance of the phosphate group. However, the phosphate group alone is not sufficient for high affinity binding to p85 C-SH2, since the pSer (**15**) and pThr (**16**) containing peptides were also inactive. Similar results have been obtained for the binding of phosphorylated peptides to the src SH2 domain.[28] The substitution of pTyr with pHO-Cha (**17**) and pHO-Tic (**18**) was not tolerated further illustrating the high specificity for a phosphorylated tyrosine residue at this position.

The inactivity of the pSer and pThr substituted analogs may be due to the three-dimensional orientation of the phosphate group of pTyr which is further removed from the Cα atom as compared to pSer and pThr. In analogs **17** and **18** the phosphate group is at a similar distance to their respective alpha carbon atom. In the crystal structures[26–29] and the solution NMR-derived structure[30] of the src SH2 domain complexed with phosphorylated peptides, the pTyr fits tightly into the binding pocket and is involved in several ionic and hydrogen bonding interactions with the protein. However, the cyclohexyl group (peptide **17**) occupies significantly more three-

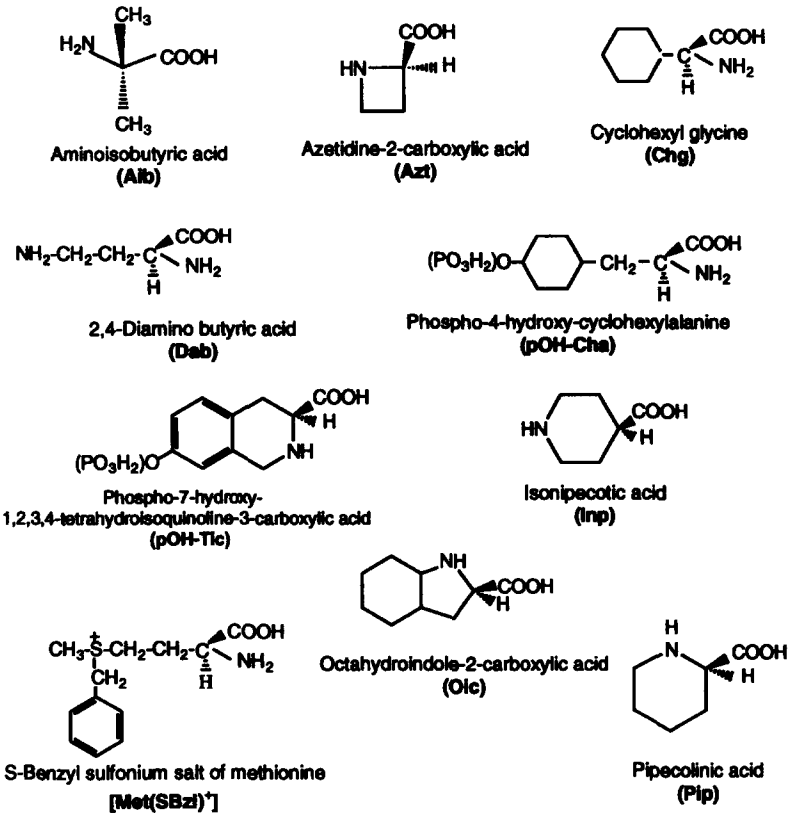

Figure 2. Structure of the unusual amino acids.

dimensional space than the planar aromatic ring of Tyr suggesting that the pTyr binding pocket is of finite volume, thus accounting for the inactivity of peptide **17**. The pOH-Tic residue (peptide **18**) has constrained conformational flexibility about the chi 1 angle, such that the aromatic side chain is restricted to either a *gauche*$^-$ (g^-) or *gauche*$^+$ (g^+) rotomer conformation.[33] This peptide (**18**) was also inactive. Peptide **18** provides three-dimensional constraints for the orientation of the phosphate group and will assist in modeling the interaction of phosphopeptides with p85 C-SH2. These studies will be reported elsewhere.

Substitutions at the pY + 1 position (Table 3)

The studies of Fantl *et al.*[19] and Songyang *et al.*[23] predicted that the preferred amino acids at the pY + 1 position should be hydrophobic (e.g. Met or Val). Charged amino acids were not well tolerated in this position.[19] We have incorporated other hydrophobic amino acids including norleucine (Nle, peptide **19**) and cyclohexylglycine (Chg, peptide **20**) (Table 3). Interestingly, in our hands, acidic amino acids [Asp, (peptide **21**) and Glu, (peptide **22**)] were also reasonably well tolerated in this position. Unlike the pY position, where a pTyr residue is critical for activity, the pY + 1 position is much more flexible with regard to the functionality of the side chain that is tolerated and maintains reasonable affinity for p85 C-SH2.

Substitutions at the pY + 2 position (Table 3)

Among the 20 naturally occurring amino acids, proline is unique due to its cyclic nature that restricts the phi angle to about –60° and is conducive to a turn conformation. In order to further probe the structural role of proline at the pY + 2 position and the potential importance of a turn conformation for binding to p85 C-SH2, several analogs incorporating unnatural cyclic amino acids were synthesized. Like proline, azetidine-2-carboxylic acid (Azt, peptide **23**) constrains the conformational space of the preceding residue due to steric interactions with the azetidine ring. However, due to the smaller size and orientation of the ring, Azt has reduced conformational effects allowing greater flexibility of the peptide backbone.[34] Incorporation of Azt (peptide **23**) for Pro led to only a 2-fold decrease in binding affinity (Table 3). Replacement with pipecolinic acid (Pip, peptide **24**) resulted in a 10-fold loss of activity. Like Pro, Pip is also capable of promoting a turn conformation and the lower affinity exhibited by this peptide may be due to the intrinsic difference in bond angles. The incorporation of isonipecotic acid (Inp, peptide **25**) favors an extended conformation and resulted in a compound that was devoid of binding affinity, suggesting that a fully extended peptide conformation may not be favorable for the binding to p85 C-SH2. Other substitutions including octahydroindole-2-carboxylic acid (Oic, peptide **26**), aminoisobutyric acid (Aib, peptide **27**) and N-methylalanine (MeA, peptide **28**) had moderate to weak binding affinity.

Substitution at the pY + 3 position (Table 3)

As previously discussed, other than the pTyr the residue that is most critical for the association of p85 C-SH2 to

PDGFR-TK is Met in the P + 3 position.[19,23] All amino acid substitutions, except for very conservative substitutions, led to a significant loss of activity. Introduction of methionine sulfoxide [Met(O), peptide **29**], methionine sulfone [$Met(O)_2$, peptide **30**] or the S-benzylsulfonium salt of methionine [$Met(SBzl)^+$, peptide **31**] abolished the binding affinity. Replacements of Met with amino acids with shorter side chains such as 2,4-diaminobutyric acid (Dab, peptide **32**) or Ser (peptide **33**) were also inactive. However, the introduction of S-methyl cysteine [Cys(SMe), peptide **34**], a lower homolog of Met, retained moderate binding affinity. Nle (peptide **35**), being the carbon homolog of Met is the only substitution that maintained significant binding affinity (~2-fold less than that of peptide **1**).

The critical nature of this residue may be deduced from the three-dimensional structure of SH2 domains complexed to phosphopeptides. The side chain of the pY + 3 amino acid residue binds in a hydrophobic pocket that is well defined by several residues in the SH2 domain and is almost completely buried as a result of these hydrophobic interactions.[27-30] Although the specific residues that interact with the pY + 3 position are different for syp, src and lck SH2 domains, in these crystal structures the pY + 3 residue is essentially anchored into the protein.[29] The p85 N-terminal and C-terminal SH2 domains both strongly prefer Met at this position.[23] It must be assumed that the structure adopted by p85 C-SH2 is quite similar to the structures adopted by the other SH2 domains (syp, src and lck), therefore, the pocket into which Met binds is hydrophobic in nature and this side chain points directly into the SH2 domain. Thus, polar and charged amino acids (peptides **29–32**) were not tolerated in this position. Also, amino acids with smaller side chains such as Ser (peptide **33**) did not bind to p85 C-SH2 at concentrations to 50 μM, possibly due to the diminished ability for hydrophobic interactions. The affinity of Cys(SMe) (**34**) and Nle (**35**) containing peptides suggests that not only the hydrophobicity of the side chain is critical but the length may also be important. The finding that the Nle substitution is an acceptable substitution for Met is consistent with other studies on p85 C-SH2.[35] From a series of linear and cyclic peptides, based on the sequence Gly-pTyr[751]-Val-Pro-Met-Leu Roller *et al.*[35] has reported that significant binding affinity was retained by peptides in which Nle was substituted for Met. Certainly, from a synthetic standpoint Nle is a desirable substitution for Met since Nle is resistant to oxidation and will not be prone to additional side reactions.[36]

Truncated analog (Table 4)

Based upon the results from the alanine and D-amino acid scans along with the tolerance of the pY + 4 position to modification we attempted to truncate peptide **1** from the C-terminus. Removal of the C-terminal residue resulted in a compound (peptide **36**) with only 10 μM binding affinity. However, neutralization of the C-terminal carboxylate with a carboxamide led to almost an 8-fold enhancement in binding affinity (peptide **37**). This suggests that the C-terminal carboxylate may be involved in unfavorable charge–charge interactions or that the carboxamide enhances hydrophobic interactions with p85 C-SH2. Similar to the results obtained in the pentapeptide series (Table 1), acetylation of the amino terminus (peptide **38**) led to a further enhancement of binding affinity (5-fold) for p85 C-SH2. This phosphorylated tetrapeptide is the smallest, potent inhibitor (IC_{50} = 0.25 μM) of the interaction of p85 C-SH2 with PDGFR-TK that has been reported. The generation of crystal and solution NMR structures of peptide **38** complexed with p85 C-SH2 will provide a starting point for the design of peptidomimetic inhibitors. These studies will be reported elsewhere.

Conclusions

A phosphorylated pentapeptide from the kinase insert region of PDGF-β receptor blocks the association of PDGF-β receptor with the C-terminal SH2 domain of PI 3-kinase with an IC_{50} of 0.67 μM. A phosphorylated tyrosine and a Met residue of the L-configuration are crucial in the pY and pY + 3 positions, respectively, for high affinity binding to p85 C-SH2. Aromaticity, the phosphate group and the correct length and orientation of the side chain were shown to be important considerations for pTyr substitutions. Neutralization of the amine and/or carboxy terminus by acetylation and/or amidation enhanced the affinity of peptide **1**. Alanine substitutions were better tolerated than D-amino acid substitutions in the pY + 1, pY + 2 and pY + 4 positions. Hydrophobic, as well as acidic amino acids were tolerated at the pY + 1 position. Several substitutions for proline at the pY + 2 position which favor reverse turn conformations led to analogs with moderate to good potency, however incorporation of Inp (peptide **25**) which favors an extended conformation was devoid of activity. Norleucine (peptide **35**) was the only substitution tolerated for Met in pY + 3 position. High affinity C-terminal truncated analogs of peptide **1** can be prepared provided that the C-terminal carboxylate is neutralized (peptide **38**). This peptide will be useful for the generation of peptidomimetic analogs.

Experimental

Materials

Peptide synthesis reagents [*N,N'*-dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole (HOBt), *N,N*-diisopropylethylamine (DIEA)] and 4-hydroxymethylphenoxymethyl (HMP) polystyrene resin were obtained from Applied Biosystems, Inc. (Foster City, CA). Fluorenylmethyloxycarbonyl (N^{α}-Fmoc)-protected amino acids on a *p*-alkoxybenzyl alcohol resin (Wang) and 4-(2',4'-dimethoxyphenyl-*N*-Fmoc-aminomethyl)-phenoxy (Rink) resins were obtained from Bachem Bioscience, Inc. (King of Prussia, PA). *N*-Methylpyrrolidone (NMP), dichloromethane (DCM) and *N,N*-dimethylformamide (DMF) were obtained from Burdick and Jackson

(Muskegon, MI). Trifluoroacetic acid (TFA) was obtained from Halocarbon (River Edge, NJ). Anisole, 1,2-ethanedithiol (EDT), indole-2-carboxylic acid, isonipecotic acid (Inp), phenol, thioanisole, di-*tert*-butyl-*N,N*-diethylphosphoramidite, 1*H*-tetrazole, 70% aqueous *tert*-butyl hydroperoxide and tetrahydrofuran (THF) were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). THF was further purified by distillation from sodium and benzophenone before use. Benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP) was obtained from NovaBiochem (LaJolla, CA). N^{α}-*t*-Butyloxycarbonyl (N^{α}-*t*-Boc) dibenzyl protected phosphate derivatives of Tyr {N^{α}-*t*-Boc-Tyr[$PO_3(Bzl)_2$]} and Ser {N^{α}-*t*-Boc-Ser[$PO_3(Bzl)_2$]} were purchased from Peninsula Laboratories, Inc. (Belmont, CA). Azetidine-2-carboxylic acid (Azt) and pipecolinic acid (Pip) were purchased from Bachem Bioscience, Inc. 1,2,3,4-Tetrahydro-7-hydroxyisoquinoline-3-carboxylic acid (pHO-Tic) was purchased from CoshiSoft/PeptiSearch (Tucson, AZ). All other protected amino acids were obtained from Applied Biosystems, Inc., Bachem Bioscience, Inc. or NovaBiochem. All amino acids were of the L-configuration unless otherwise noted.

Octahydroindole-2-carboxylic acid (Oic) was prepared from indole-2-carboxylic acid as previously reported.[37] The N^{α}-9-fluorenylmethoxycarbonyl (N^{α}-Fmoc) derivatives of Cys(SMe), pHO-Tic, Azt, Pip, Oic and Inp were prepared from the corresponding amino acids using fluorenylmethyl chloroformate.[38]

Preparation of N^{α}-t-*Boc-4-hydroxycyclohexyl alanine* (N^{α}-t-*Boc-pHO-Cha)*

Catalytic hydrogenation of N^{α}-*t*-Boc-Tyr using PtO_2/CH_3COOH in methanol yielded N^{α}-*t*-Boc-cyclohexyl alanine (N^{α}-*t*-Boc-Cha) and N^{α}-*t*-Boc-pHO-Cha. N^{α}-*t*-Boc-pHO-Cha was purified using reversed-phase high performance liquid chromatography (RP-HPLC) and was subsequently used for the synthesis of peptide **17**.

Peptide synthesis

Peptides were synthesized on an automated peptide synthesizer (Applied Biosystems 430A or 431A) utilizing standard solid phase techniques (SPPS) for Fmoc protected amino acids on a Wang or Rink resin.[39,40] Syntheses were performed on a 0.25 mmole scale using a DCC/HOBt and/or a BOP/HOBt/DIEA coupling strategy. The side chain protecting groups were *t*-Boc for 2,4-diaminobutyric acid (Dab), *t*-butyl for Ser and trityl (Trt) for cysteine. Peptides **2** and **4** were acetylated using acetylimidazole/DMF after chain assembly. The N-terminal phosphotyrosine residue was incorporated during chain assembly using N^{α}-*t*-Boc-Tyr[$PO_3(Bzl)_2$][41,42] or the N^{α}-Fmoc-Tyr(PO_3H_2)[43] derivative. For peptide **15**, phosphoserine was incorporated as the N^{α}-*t*-Boc-Ser[$PO_3(Bzl)_2$] derivative. Utilization of the N^{α}-*t*-Boc-Tyr[$PO_3(Bzl)_2$] or N^{α}-*t*-Boc-Ser[$PO_3(Bzl)_2$] derivatives in methionine containing peptides led to a major byproduct during deprotection which corresponded to the S-benzyl sulfonium salt of methionine.[36] The desired phosphopeptide was obtained from the S-benzyl sulfonium salt by catalytic hydrogenation with 20% Pd/C in methanol at room temperature for 30 min. Other phosphopeptides including the phosphothreonine containing analog (peptide **16**) were synthesized utilizing a global phosphorylation strategy in which the free hydroxyl of Tyr was phosphitylated using di-*tert*-butyl-*N,N*-diethylphosphoramidite and oxidized with 70% aqueous *tert*-butyl hydroperoxide while still anchored to the resin.[42,44,45]

Peptides were cleaved from the resin using either a mixture of 82.5% TFA, 5.0% phenol, 5.0% H_2O, 5.0% thioanisole, 2.5% EDT (Reagent K)[46] or 90.0% TFA, 5.0% thioanisole, 3.0% EDT, 2.0% anisole (Reagent R)[47] for 3 h at room temperature. For peptides **29** and **30**, a mixture of 95.0% TFA and 5.0% H_2O was used for the cleavage. After filtration, the TFA was removed under reduced pressure and the peptides were precipitated from diethyl ether, filtered, solubilized in aqueous solution and lyophilized. The crude peptides were purified by RP-HPLC using a C18 column (Vydac 218TP1022) with a linear gradient of 0–40% B over 90 or 120 min (A = 0.1% TFA/H_2O; B = 0.1% TFA/CH_3CN). Analytical RP-HPLC was performed on a Vydac 218TP54 (0.46 × 25-cm) column using a linear gradient of 0–66% B over 22 min. The structural integrity of the purified peptides was determined by amino acid analysis, ^{1}H-NMR, ^{31}P NMR (DMSO-d_6, Varian Unity 400 MHz spectrometer, Palo Alto, CA) and electrospray (ES) mass spectrometry (Finnigan MAT-900Q, Bremen, Germany). Positive ion electrospray ionization (ESI) samples were dissolved in a water/methanol solution acidified with 2.5% v/v acetic acid and infused through the ESI source at a flow rate of 1 μL min^{-1}. Values found were in agreement with the theoretical values (Table 5).

Expression and purification of the C-terminal SH2 domain of the p85 subunit of PI3-kinase-GST fusion protein (GST p85 C-SH2)

The pGEX plasmid expressing the GST p85 C-SH2 fusion protein was used in these studies. Both the expression and purification of the fusion protein were performed as previously described.[48] To prepare the [^{35}S]GST p85 C-SH2 fusion protein, a 75 mL overnight culture of *Escherichia coli* expressing the GST p85 fusion protein was added to 1.0 L of LB broth containing 100 μg mL^{-1} ampicillin. The cultures were incubated at 37 °C until reaching a density of A_{600} = 1.0. Isopropyl thio-β-D-galactoside (1 mM) was added and 15 min later, 10 mCi of *trans*^{35} S-label was added and cultures incubated for an additional 3 h at 37 °C. The cells were then lysed by sonication and fusion proteins purified by affinity chromatography using glutathione–agarose (CL-4B) beads.

Table 5. Analytical data for the synthetic peptides

Peptide	RP-HPLC		ESMS	
	R_t (min)*	Purity(%)†	Calculated	Found
1	14.0	> 99	701.78	702.2
2	17.7	> 98	743.82	744.0
3	15.8	> 98	700.80	700.5
4	17.1	> 98	742.86	742.4
5	16.1	> 98	673.73	673.5
6	13.1	> 99	675.74	676.4
7	15.2	> 95	641.70	641.3
8	13.8	> 99	659.70	659.4
9	17.8	> 98	701.78	701.8
10	14.2	> 99	701.78	702.5
11	14.5	> 99	701.78	702.5
12	17.8	> 98	701.78	701.6
13	17.4	> 98	701.78	701.6
14	14.8	> 99	621.80	622.4
15	16.9	> 76‡	625.68	625.6
16	13.8	> 99	639.73	640.6
17	17.4	> 99	707.83	707.5
18	14.0	> 99	713.79	714.2
19	18.9	> 98	715.81	716.5
20	19.4	> 95	741.85	741.9
21	12.7	> 97	717.50	718.5
22	12.8	> 99	731.77	732.4
23	13.4	> 99	687.80	687.4
24	14.9	> 99	715.81	715.5
25	13.6	> 98	715.81	716.4
26	15.7	> 99	755.87	756.4
27	14.8	> 99	689.77	690.4
28	14.2	> 99	689.77	690.4
29	15.3	> 98	717.78	717.5
30	15.9	> 98	733.80	733.5
31	17.8	> 94	791.78	792.4
32	15.2	> 98	670.73	670.6
33	15.2	> 95	657.77	658.3
34	17.0	> 98	687.75	687.5
35	18.6	> 97	683.75	684.6
36	13.2	> 99	588.20	590.0
37	10.4	> 99	587.64	589.1
38	11.5	> 99	629.64	630.3

*For analytical HPLC conditions, see experimental conditions.
†Based upon analytical HPLC at 214 nM.
‡This compound was 76% pure by analytical HPLC with one unidentified contaminant, but the major component is the indicated compound and since it is inactive, it has been included for discussion purposes.

Preparation of the intracellular tyrosine kinase domain of the PDGF-β receptor

Lysates from SF9 insect cells expressing the PDGFR-TK were incubated with M2 affinity beads and the complexes were washed several times with Tris buffer containing protease inhibitors and sodium orthovanadate. Complexes were centrifuged and resuspended in HEPES buffer containing 1 mM ATP, 10 mM $MnCl_2$ and 5 mM $MgCl_2$ to stimulate phosphorylation of the PDGFR-TK.

GST p85 C-SH2–PDGFR-TK binding assay

Binding of [^{35}S]GST p85 C-SH2 fusion proteins to the phosphorylated PDGFR-TK was assayed in 20 mM HEPES buffer containing 10 μg mL^{-1} of each of the protease inhibitors, phenylmethylsulfonyl fluoride (PMSF), pepstatin, leupeptin and aprotinin, along with 0.5 mM EDTA and 0.1% NP-40. The binding assays were performed in 96 well Millipore filter plates in a final volume of 250 μL of HEPES buffer containing 135 μL phosphorylated PDGFR-TK beads complex (1 μg receptor/well), 10 μL [^{35}S]GST p85 C-SH2 fusion protein (30,000 cpm well^{-1}) and 5 μL of peptide inhibitor as indicated. Samples were incubated at 25 °C for 20 min with continuous rocking. Binding was terminated by filtration through the filter plates using a Millipore multiscreen filtration manifold. Filter plates were washed 4 times with 150 μL HEPES buffer followed by the addition of 30 μL Hi-load scintillant. The radio-

activity that was retained on the filters was counted in a Wallac 1450 Microbeta counter. Total binding was defined as [^{35}S]GST p85 C-SH2 fusion protein bound to the PDGFR-TK bead complex retained on the filter plates. Specific binding was defined as total binding minus nonspecific binding. IC_{50} values were calculated by weighted nonlinear regression curve fitting.

Acknowledgements

We thank Dana DeJohn and Joseph Loo for the mass spectral data. We also thank Carmen Faraianu and Brian Tobias for the NMR spectral data and Scott Buckel for the amino acid analyses data.

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(*Received in U.S.A.* 13 *March* 1995; *accepted* 29 *April* 1995)