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NORLEUCINE AS A REPLACEMENT FOR METHIONINE IN PHOSPHATASE-RESISTANT LINEAR AND CYCLIC PEPTIDES WHICH BIND TO P85 SH2 DOMAINS

Peter P. Roller, Akira Otaka, Motoyoshi Nomizu,
Mark S.Smyth, Joseph J. Barchi, Jr. and Terrence R. Burke, Jr.*

Laboratory of Medicinal Chemistry, Bldg. 37, Rm. 5C02, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD 20893

and

Randi D. Case, Gert Wolf and Steven E. Shoelson

Joslin Diabetes Center & Department of Medicine, Harvard Medical School, Boston, MA 02215

Abstract: A Met residue in the pTyr+3 position has previously been shown to be an important determinant for high affinity binding of peptides to PI 3-kinase p85 SH2 domains. In the present work, a series of linear and cyclic peptides based on the sequence "Gly-pTyr-Val-Pro-Met-Leu" as well as analogues having pTyr replaced by the phosphatase-resistant pTyr mimetics, phosphonomethyl phenylalanine (Pmp) or difluorophosphonomethyl phenylalanine (F_2Pmp), were synthesized and their binding potency in p85 SH2 domain preparations compared with corresponding peptides in which the Met has been substituted by Nle. Nle is a chemically more stable, isosteric Met homologue in which the sulfur has been replaced by a methylene. Significant binding potency was retained by the Nle-containing peptides, indicating that Met is not absolutely essential for high affinity binding to this SH2 domain.

Pharmacological modulation of growth factor and cytokine signalling pathways potentially offers a new approach toward the treatment of several diseases, including certain forms of cancer[1]. Protein-tyrosine kinases (PTKs) are key mediators in these pathways, and function by phosphorylating tyrosyl residues within protein substrates. Subsequent PTK signal transduction depends on the high affinity association of secondary signalling molecules to the newly generated phosphotyrosyl (pTyr)-bearing proteins. This recognition and binding is mediated by homologous protein modules termed "src homology 2" (SH2) domains[2,3] and the inhibition of specific SH2 domain binding interactions has become a target for new drug design[4,5].

Phosphatidylinositol 3-kinase (PI 3-kinase), an enzyme which generates second messengers in a number of PTK-dependent pathways, is heterodimeric and contains an 85 kilodalton (p85) non-catalytic subunit in addition to a 110 kilodalton (p110) catalytic subunit[6]. The p85 protein has two SH2 domains which allow it to recognize and bind to specific pTyr-bearing proteins, and in so doing both translocate and activate the p110 subunit. Evidence suggests that disruption of PI 3-kinase binding can attenuate mitogenic signal transduction by some PTKs[7,8] and we have therefore undertaken efforts to develop inhibitors of PI 3-kinase p85 SH2 binding[9,10].

Our starting point in this endeavor was the previous discovery that small (5-6 amino acid) peptides bearing the general sequences pTyr-Met-Xxx-Met or pTyr-Val-Xxx-Met could effectively compete with larger pTyr peptides and proteins for binding to the p85 SH2 domains[8,11]. Since pTyr-containing peptides are enzymatically inactivated by both protein-tyrosine phosphatases (PTPs) and peptidases, we developed phosphonate-based pTyr mimetics which allowed the replacement of the pTyr residue with phosphatase-resistant analogues while retaining SH2 domain binding potency[9,12-17].

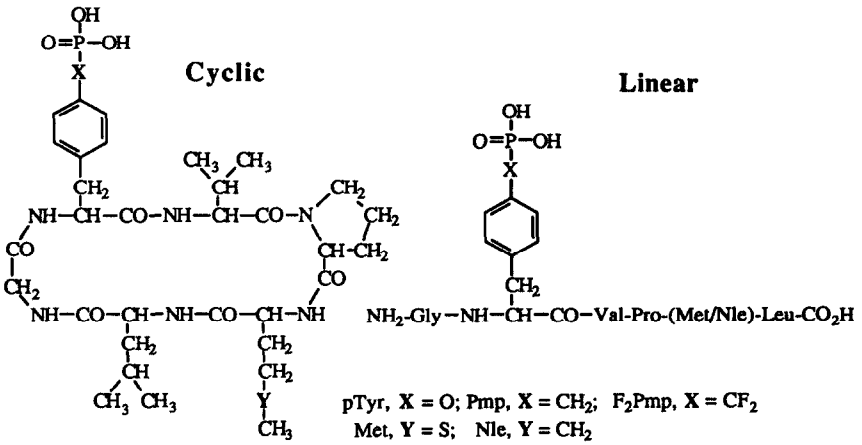

Figure 1. Structures of peptides, including phosphatase-resistant pTyr mimetics Pmp and F_2Pmp.

To reduce susceptibility to proteases and to restrict conformational flexibility as an aid in peptidomimetic design, we recently reported cyclizing these peptides[10]. The cyclic hexameric peptides were of the general structure, cyclo[Gly-Xxx-Val-Pro-Met-Leu], where Xxx is either pTyr or a phosphatase-resistant mimetic, such as phosphonomethyl phenylalanine (Pmp) or difluorophosphonomethyl phenylalanine (F_2Pmp). These cyclic peptides retained significant binding potency in p85 SH2 domain assays, and are currently being studied by 500 MHz NMR to define active conformations[18]. In our NMR studies it was noted that rapid conversion of the Met residue to its sulfoxide occurred in DMSO solution. This phenomenon is illustrative of the ease with which Met residues are oxidized, and is consistent with difficulties encountered in synthesizing Met-containing peptides, where it is often seen that Met residues undergo alkylation and oxidation during peptide chain assembly or deprotection.

Previous reports have indicated the importance of a Met residue in the pTyr+3 position for binding to p85 SH2 domains[19]. This is consistent with a more general finding that residues in the C-terminal proximity to the pTyr are critical determinants for differential binding amongst various families of SH2 domains[20]. X-ray analysis of high affinity pTyr-peptides bound to both the src and p56lck SH2 domains show that the pTyr+3 residues bind within well defined pockets[21,22]. By analogy, it could be postulated that other SH2 domains, including p85, may have well defined binding regions for critical recognition residues.

In light of this, we were interested in determining whether replacement of the easily oxidized pTyr+3 Met residue by its nonsulfur-containing isostere norleucine (Nle)[23] would result in retention of binding affinity in a p85 SH2 domain binding system. A series of linear and cyclic peptides were therefore prepared incorporating either pTyr or a phosphatase-resistant mimetic and either a Met or Nle residue at the pTyr+3 position[24] (Figure 1). Peptides were examined for their ability to bind with a C-terminal p85 SH2 domain GST fusion protein[25]. Binding curves are shown in Figure 2 with tabulated ID_{50} values listed in Table 1.

Nle-containing peptides, while being less potent than their Met analogues, did retain significant affinity. Of note is the high potency of linear peptide **2b** (ID_{50} = 1.6 μM), which has both phosphatase-resistant phosphonomethyl phenylalanine (Pmp) and Nle in place of pTyr and Met, respectively. In the cyclic series, peptide **6b** (ID_{50} = 8.9 μM), while being fourfold less potent than its linear counterpart **6a** (ID_{50} = 2.24 μM), still exhibited good affinity.

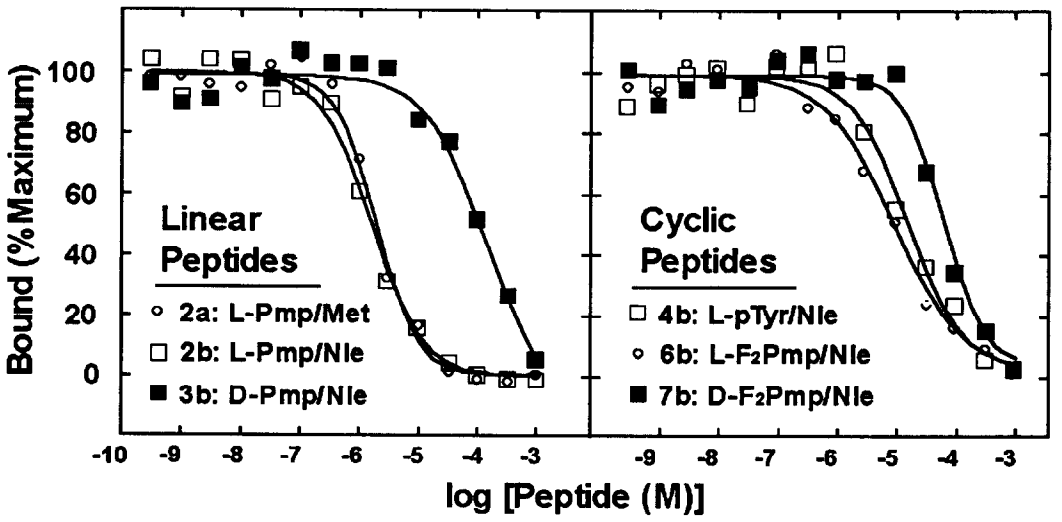

Figure 2. Competitive binding of selected peptides with the C-terminal SH2 domain of p85. Identification of peptides is provided in Table 1.

		ID_{50} μM	
		(a) Xxx = Met	(b) Xxx = Nle
1	Gly-pTyr-Val-Pro-Xxx-Leu	0.17 ± 0.02[a]	N.D.
2	Gly-Pmp-Val-Pro-Xxx-Leu	0.98 ± 0.09[a]	1.6 ± 0.2
3	Gly-(D-Pmp)-Val-Pro-Xxx-Leu	29.4 ± 9.2[a]	109 ± 59
4	cyclo[Gly-pTyr-Val-Pro-Xxx-Leu]	1.01 ± 0.15[b]	15.6 ± 4.6
5	cyclo[Gly-Pmp-Val-Pro-Xxx-Leu]	5.2 ± 0.7[b]	N.D.
6	cyclo[Gly-F_2Pmp-Val-Pro-Xxx-Leu]	2.24 ± 0.4[b]	8.9 ± 2.0
7	cyclo[Gly-(D-F_2Pmp)-Val-Pro-Xxx-Leu]	32.4 ± 5.5[b]	59.1 ± 10.3

Table 1. Peptides and their associated ID_{50} values in assays conducted against C-terminal p85 SH2 preparations. Structures of cyclic peptides and phosphatase-resistant analogues Pmp and F_2Pmp are shown in Figure 1. [a]Values have been reported previously[9]. [b]Values have been reported previously[10].

Somewhat surprisingly, F_2Pmp-containing cyclic peptide **6b** was nearly twice as potent as its corresponding cyclic pTyr-containing parent **4b** (ID_{50} = 15.6 μM). This may indicate that conformational constraints induced by cyclization prevent optimum binding by residues occupying the pTyr and pTyr+3 positions. In this example, perhaps a slightly more favorable orientation is afforded the F_2Pmp residue relative to the pTyr residue. Finally, D-Pmp or D-F_2Pmp containing peptides (**3** and **7**) were much less potent than their L-counterparts (**2** and **6**, respectively).

In summary, significant binding potency to the C-terminal p85 SH2 domain can be exhibited by peptides containing Nle at the pTyr+3 position. Phosphatase resistant Nle-containing linear and cyclic peptides such as **2b** and **6b** are more easily synthesized and exhibit greater stability than their Met counterparts, and may be useful in the development of p85 SH2 domain-directed inhibitors.

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