# Nonhydrolyzable Phosphotyrosyl Mimetics for the Preparation of Phosphatase-Resistant SH2 Domain Inhibitors†

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ABSTRACT: Src homology 2 (SH2) domains participate in protein tyrosine kinase (PTK)-mediated cellular signal transduction through their ability to bind with high affinity to phosphotyrosyl (pTyr)-bearing protein sequences. Although peptides containing pTyr competitively inhibit the binding between phosphoproteins and cognate SH2 proteins in a sequence-specific manner, such peptides are rapidly dephosphorylated by cellular phosphatases. We now describe our efforts to develop SH2 inhibitory peptides containing phosphatase-resistant pTyr surrogates. The parent compound, (phosphonomethyl)phenylalanine (Pmp), is a phosphonate-based mimetic of pTyr in which the phosphate ester oxygen (>$COPO_3H_2$) has been replaced by a methylene unit (>$CCX_2PO_3H_2$, $X_2 = H_2$). Pmp analogues bearing fluorine ($X_2$ = H, F or $X_2 = F_2$) or hydroxyl ($X_2$ = H, OH) substituents on the phosphonate $\alpha$-methylene carbon have been prepared and incorporated into peptides for use as SH2 domain inhibitors. In an assay using the C-terminal SH2 domain of phosphatidylinositol (PI) 3-kinase, peptides having a GXVPML sequence [where X = pTyr, Pmp, hydroxy-Pmp (HPmp), monofluoro-Pmp (FPmp), and difluoro-Pmp ($F_2$Pmp)] exhibited binding potency in the order HPmp < Pmp < FPmp < $F_2$Pmp = pTyr. Distinct peptide sequences which bind selectively with Src and Grb2 SH2 domains were also prepared with pTyr and $F_2$Pmp. The $F_2$Pmp peptides bound with high (0.2- to 5-fold) relative affinity, compared to analogous pTyr peptides. We conclude that peptides containing $F_2$Pmp bind to SH2 domains with high affinity and specificity and, being resistant to cellular phosphatases, should provide a generally useful tool for disrupting SH2 domain-mediated signaling pathways in intact cells.

SH2 domains are phosphotyrosine-binding modules found in a variety of important signal-transducing molecules, where they mediate protein–protein associations with protein tyrosine kinases (PTK) and their cellular substrates (Pawson & Schlessinger, 1993; Panayotou & Waterfield, 1993). The central roles played by PTKs in mitogenic signaling cascades and the involvement of aberrant or overexpressed PTKs in several cancers and proliferative diseases have made the development of inhibitors which block SH2 domain binding desirable both as biological tools and as potential therapeutic agents (Brugge, 1993; Burke, 1992). Phosphorylation at particular tyrosine residues serves as an on–off switch for SH2 domain binding, while the residues surrounding phosphotyrosine (pTyr) confer a secondary level of specificity. By this mechanism different SH2 proteins interact with distinct binding sites on target proteins.

Phosphopeptides modeled after these target sequences also bind to SH2 domains with high affinity and moderate selectivity, thereby providing potential means for inhibiting specific SH2 domain-mediated signaling pathways (Escobedo et al., 1991; Fantl et al., 1992; Felder et al., 1993; Payne et al., 1993; Piccione et al., 1993; Songyang et al., 1993). The hydrolytic lability of tyrosine phosphate to cellular PTPs led us to examine phosphatase-resistant amino acid analogues which could serve as pTyr mimetics in SH2 binding interactions. (Phosphonomethyl)phenylalanine (Pmp) is a phosphonate homologue of pTyr in which the phosphate ester oxygen has been replaced by a methylene, and we initially developed a protected form of Pmp suitable for incorporation into peptides by solid-phase synthesis (Burke et al., 1991; Shoelson et al., 1991). We previously showed that a Pmp-containing peptide modeled on the sequence surrounding Tyr315 of the polyoma virus middle T antigen (mT) blocked the *in vitro* association between the c-Src/mT complex and phosphatidylinositol (PI) 3-kinase. A 5-fold higher concentration of Pmp-peptide was required for inhibiting this phosphoprotein–SH2 protein interaction than the corresponding phosphopeptide (Domchek et al., 1992), although in both cases the peptides acted as competitive inhibitors by binding directly to PI 3-kinase (p85) SH2 domains. Decreased affinity of the Pmp-peptide might be due either to a higher $pK_{A2}$ of the phosphonate (7.1) relative to pTyr (5.7) (Domchek et al., 1992) or to loss of hydrogen bonding between the phosphate ester oxygen and the SH2 domain (Waksman et al., 1992, 1993; Eck et al., 1993). To address both of these possibilities, we designed new analogues of Pmp which bore fluorine substituents on the methylene bridge (Burke et al., 1993a). Fluorine substituents $\alpha$ to the phosphonate have the double effect of lowering the phosphonate $pK_{A2}$ and introducing hydrogen-bonding interactions similar to oxygen. In model

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benzylic phosphonate systems we observed that $pK_{A2}$ values were lowered approximately one unit per fluorine added, with the $\alpha,\alpha$-difluorobenzyl phosphonate ($pK_{A2}$ = 5.7) having a value lower than that of benzyl phosphate itself ($pK_{A2}$ = 6.2) (Smyth et al., 1992). We therefore recently developed mono- and difluoro-Pmp analogues suitably protected for incorporation into peptides by solid-phase synthesis (Burke et al., 1993a,b; Otaka et al., 1993; Smyth & Burke, 1994). In the current studies we have incorporated various substituted Pmp derivatives into peptides and determined relative effects on SH2 domain binding.

## EXPERIMENTAL PROCEDURES

*Peptide Synthesis.* All peptides were prepared following a stepwise, $N^{\alpha}$-Fmoc strategy. Phosphopeptides were synthesized with $N^{\alpha}$-Fmoc-*O*-(*O,O*-dimethoxyphosphoryl)-L-tyrosine (Piccione et al., 1993; Nomizu et al., 1994). Peptides containing racemic (phosphonomethyl)phenylalanine (Pmp), (phosphonohydroxymethyl)phenylalanine (HPmp), and (phosphonofluoromethyl)phenylalanine (FPmp) were synthesized with $N^{\alpha}$-Fmoc-4-[(*O,O*-di-*tert*-butyl phosphono)methyl]phenylalanine (Shoelson et al., 1991; Domchek et al., 1992), $N^{\alpha}$-Fmoc-4-[(*O,O*-di-*tert*-butyl phosphono)hydroxymethyl]phenylalanine (Burke et al., 1993a), and $N^{\alpha}$-Fmoc-4-[(*O,O*-di-*tert*-butyl phosphono)fluoromethyl]phenylalanine (Burke et al., 1993a), respectively. Peptides containing racemic (phosphonodifluoromethyl)phenylalanine ($F_2$Pmp) and optically pure L-$F_2$Pmp were prepared with the appropriate $N^{\alpha}$-Fmoc-4-[(*O,O*-diethyl phosphonodifluoromethyl]phenylalanine derivatives (Burke et al., 1993b; Smyth & Burke, in press), using manual SPPS methodology and deprotection procedures published recently (Otaka et al., 1993). Peptides were purified and diastereomers were separated by HPLC. All peptides provided mass spectra and amino acid analyses consistent with their expected structures.

*SH2 Domain Binding Assays.* Details of the SH2 domain competition assay have been published previously (Piccione et al., 1993). In this study three distinct assays were used to determine relative SH2 domain affinities for phosphonopeptides vs the corresponding phosphopeptides. In each assay a glutathione S-transferase (GST)/SH2 domain fusion protein was paired with an appropriate high-affinity [$^{125}$I]Bolton-Hunter-radiolabeled phosphopeptide, and varying concentrations of unlabeled peptides were added as competitors. The C-terminal SH2 domain of PI 3-kinase p85 was paired with IRS-1 pY628, GNGDpYMPMSPKS (Piccione et al., 1993); the Src SH2 domain was paired with hmT pY324, KEPQpYEEIPIYL (Payne et al., 1993); and the Grb2 SH2 domain was paired with Shc pY317, ELFDDPSpYVN-VQNLDK (G. Wolf, R. Case, and S. E. Shoelson, manuscript in preparation). An underline denotes the position of the [$^{125}$I]Bolton-Hunter-modified lysine.

GST/SH2 domain fusion proteins (0.5–1.0 $\mu$M, estimated by Bradford assay), 35 fmol of HPLC-purified, [$^{125}$I]Bolton-Hunter-treated phosphopeptide (67 nCi), and varying concentrations of unlabeled peptides were combined in a 200-$\mu$L total volume of 20 mM Tris-HCl, 250 mM NaCl, 0.1% bovine serum albumin, and 10 mM dithiothreitol, pH 7.4, and vortexed. Glutathione–agarose (25 $\mu$L of a 1:4 aqueous slurry, Molecular Probes) was added, and the samples were incubated overnight at 22 °C with constant mixing. Following centrifugation for 5 min at 12000*g*, supernatant solutions were removed by aspiration and [$^{125}$I]radioactivity associated with the unwashed pellets was determined with a $\gamma$-counter. Analyses of the binding data were as previously reported (Piccione et al., 1993; Case et al., 1994).

*Phosphatase Treatment.* Peptides Gly-pTyr-Val-Pro-Met-Leu and Gly-$F_2$Pmp-Val-Pro-Met-Leu were labeled with [$^{125}$I]-Bolton-Hunter reagent and purified by HPLC as described (Piccione et al., 1993; Case et al., 1994). The radiolabeled peptides were dissolved in 50 $\mu$L of 50 mM HEPES buffer (pH 7.1) containing 50 mM NaCl, 10 mM DTT, and 2 mM EDTA. An equal volume of a 1:1 slurry of alkaline phosphatase–agarose (Sigma) was added, and the mixture was incubated overnight at room temperature. Supernatant solutions were removed and incubated with the varying concentrations of C-terminal p85 SH2/GST fusion protein and glutathione–agarose as described above for the SH2 domain assays. Following an overnight incubation the bound peptides were separated by centrifugation.

## RESULTS

*SH2 Domain Inhibitor Design.* Phosphorylated peptides having appropriate sequences block *in vitro* associations between intact phosphoproteins and SH2 domain-containing proteins due to competition between phosphoprotein and phosphopeptide for SH2 domain occupancy (Escobedo et al., 1991; Auger et al., 1992; Fantl et al., 1992). To inhibit related interactions in cells, we wanted to prepare peptides that were resistant to cellular phosphatases and proteases. The major subject of this paper relates to prevention of phosphorolysis. We further reasoned that short peptide sequences would provide a smaller targets for endoproteases, and chemical blockage of the N- and C-termini would completely block the actions of exoproteases. We have previously determined that for p85 SH2 domains the shortest sequence which binds with full potency is a six amino acid peptide having one residue N-terminal and one residue C-terminal to the critical YM/VXM motif (E. Piccione, R. Case, and S. Shoelson, manuscript in preparation). On the basis also of crystal structure data for Src and Lck SH2 domain/phosphopeptide complexes (Eck et al., 1993; Waksman et al., 1993), and the supposition that additional SH2 domains might function similarly, all peptides in the current studies contain six residues. Sequences based on those surrounding Tyr751 of the PDGF receptor, Tyr324 of the hamster polyoma virus middle T antigen, and Tyr895 of IRS-1 were chosen for inhibition of PI 3-kinase (Piccione et al., 1993), Src (Songyang et al., 1993; Payne et al., 1993), and Grb2 (Sun et al., 1993; G. Wolf, R. Case, and S. E. Shoelson, manuscript in preparation) SH2 domains, respectively. In most cases the N- and C-termini of the peptides were blocked with acetyl and carboxamido groups, respectively.

*Phosphonopeptide Binding with the PI 3-Kinase p85 C-SH2 Domain.* We have shown previously that peptides which contain pTyr-Met/Val-Xxx-Met motifs bind with highest affinity to the SH2 domains of PI 3-kinase p85 (Piccione et al., 1993; E. Piccione, R. Case, and S. Shoelson, manuscript in preparation). We now show (Figure 1) that in this assay the hexapeptide H-Gly-pTyr-Val-Pro-Met-Leu-OH binds with high affinity to the p85 C–SH2 domain ($ID_{50}$ = 0.17 $\mu$M). The corresponding unphosphorylated peptide does not bind ($ID_{50}$ >1000 $\mu$M), demonstrating that phosphorylation is crucial for the SH2 domain interaction. Phosphonopeptides having the identical sequence were similarly studied. The unsubstituted Pmp peptide bound with an $ID_{50}$ value of 1.0 $\mu$M, a 6-fold lower relative affinity than the corresponding pTyr peptide. Hydroxyl substitution of the phosphonate methylene (HPmp) reduced affinity ($ID_{50}$ = 3.3 $\mu$M; 20-fold lower than pTyr), whereas monofluorine substitution (FPmp) enhanced Pmp binding affinity ($ID_{50}$ = 0.5 $\mu$M; 3-fold lower relative affinity than pTyr). A peptide

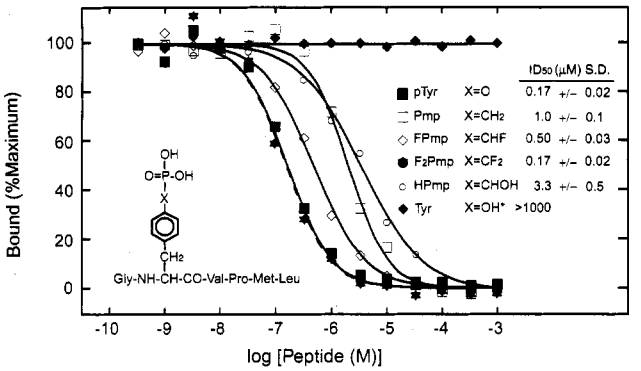


FIGURE 1: Competitive binding of peptides with the C-terminal SH2 domain of p85. Binding assays were conducted as described under Experimental Procedures. The structures of competing peptides (Gly-Xxx-Val-Pro-Met-Leu) are indicated at the lower left; all residues are in the L-configuration and Xxx = phosphotyrosine (■), (phosphonomethyl)phenylalanine (Pmp, □), monofluoro-Pmp (◊), difluoro-Pmp (★), hydroxy-Pmp (○), and unsubstituted tyrosine (♦). $ID_{50}$ values (μM) are displayed at the right. *For tyrosine, X = OH without an attached phosphate or phosphonate.

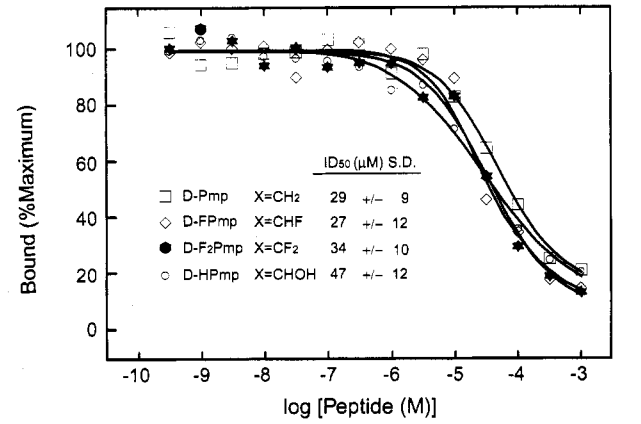


FIGURE 2: Competitive binding assays of D-configured peptide phosphonates with the C-terminal SH2 domain of p85. The sequence of each peptide is Gly-Xxx-Val-Pro-Met-Leu, where Xxx = D-Pmp (□), D-monofluoro-Pmp (◊), D-difluoro-Pmp (★), or D-hydroxy-Pmp (○). $ID_{50}$ values (μM) are displayed.

substituted with $F_2$Pmp bound to the C–SH2 domain of p85 with the same affinity as the corresponding phosphopeptide ($ID_{50}$ = 0.17 μM). Therefore, for binding with the p85 C–SH2 domain peptide affinities can be ranked: pTyr = $F_2$Pmp > FPmp > Pmp > HPmp ≫ Tyr. Relative affinities $F_2$Pmp > FPmp > Pmp follow the order of net electronegativity of substituents at the phosphonate carbon ($F_2$ > F > H) to support our previous suggestion that phosphonate side chain $pK_A$ might be important for SH2 domain binding affinity (Domchek et al., 1992). This is also consistent with our previous studies using model benzylic phosphonates, in which we observed that $pK_{A2}$ values were lowered by approximately one unit per fluorine added (Smyth et al., 1992). On the basis of electrostatic effects alone, hydroxyl substitution might be expected to slightly decrease side-chain $pK_A$ and thereby increase binding affinity. The finding that the HPmp peptide binds with lower relative affinity to the SH2 domain suggests the possibility of superimposed steric effects with hydroxyl but not fluoro substituents.

Binding was highly stereoselective for L-enantiomers, as corresponding D-Pmp peptides were much less potent (Figure 2). Furthermore, $ID_{50}$ values for peptides containing substituted D-Pmp analogues ranged from 27 to 47 μM, suggesting that, in addition to having decreased affinity, discrimination between Pmp, HPmp, FPmp, and $F_2$Pmp was lost.

*$F_2$Pmp-peptide Binding with p85, Src, and Grb2 SH2 Domains.* To establish the generality of $F_2$Pmp as a pTyr mimetic in peptides, we were interested in designing selective inhibitors of alternative SH2 domains. We have previously determined peptide motifs which bind with high affinity and selectivity with a wide variety of SH2 domains (Domchek et al., 1992; Felder et al., 1993; Payne et al., 1933; Piccione et al., 1993; Songyang et al., 1993, 1994; Case et al., 1994). This information was used in the design of potential inhibitors of the SH2 domains of Src and Grb2.

To provide a uniform comparison of inhibitory potencies among PI 3-kinase, Src, and Grb2 SH2 domains, all peptides were prepared as carboxamides with N-terminal capping by acetylation. Results in the PI 3-kinase p85 assay using acetyl-Asp-Xxx-Val-Pro-Met-Leu-$NH_2$ (Xxx = pTyr or $F_2$Pmp) (Figure 3A) were consistent with those previously obtained using the uncapped sequence (Figure 1), the $F_2$Pmp-bearing peptide being equipotent to the phosphopeptide. For the Src SH2 domain, peptides incorporating a pTyr-Glu-Glu-Ile motif bind with high affinity (Payne et al., 1993; Songyang et al., 1993). Six residue peptides having the sequence acetyl-Gln-Xxx-Glu-Glu-Ile-Pro-$NH_2$ were tested for SH2 domain binding (Xxx = pTyr or $F_2$Pmp); results are shown Figure 3B. The pTyr peptide binds with an $ID_{50}$ value of 5.7 μM, whereas the corresponding $F_2$Pmp peptide bound with approximately 5-fold higher relative affinity ($ID_{50}$ = 1.0 μM). The Grb2 SH2 domain binds with high affinity to sequences containing Asn at the pTyr +2 position (Songyang et al., 1993, 1994). Such sites are found within Shc, the EGF receptor, and IRS-1, for example, and the corresponding peptides bind with equivalently high relative affinities (G. Wolf, R. Case, and S. E. Shoelson, manuscript in preparation). Peptides in the current study having the sequence acetyl-Asn-Xxx-Val-Asn-Ile-Glu-$NH_2$ were modeled on the site surrounding IRS-1 Tyr895 (Sun et al., 1991). The pTyr peptide bound with an $ID_{50}$ value of 0.9 μM, vs a value of 4.7 μM for the corresponding $F_2$Pmp peptide (Figure 3C). The corresponding peptide containing the D-stereoisomer of $F_2$-Pmp binds with much lower relative affinity. Therefore, in each case tested peptides containing $F_2$Pmp bind with high affinity to the appropriate SH2 domains. In some cases $F_2$-Pmp peptides bind with slightly higher relative affinity than corresponding pTyr peptides, while in other cases $F_2$Pmp peptide affinities are slightly lower.

In every case tested, $F_2$Pmp-substituted peptides also retain the same SH2 domain selectivity as the parent phosphopeptides when tested in heterologous SH2 domain assays. For example, the peptides which bind with high affinity with Src and PI 3-kinase p85 SH2 domains were also analyzed in the Grb2 SH2 domain assay. Neither the peptides which bind with Src SH2 domain (acetyl-Gln-pTyr-Glu-Glu-Ile-Pro-$NH_2$, acetyl-Gln-$F_2$Pmp-Glu-Glu-Ile-Pro-$NH_2$) nor those that bind with high affinity with PI 3-kinase p85 SH2 domains (acetyl-Asp-pTyr-Val-Pro-Met-Leu-$NH_2$, acetyl-Asp-$F_2$Pmp-Val-Pro-Met-Leu-$NH_2$) exhibited observable binding with the Grb2 SH2 domain (in each case $ID_{50}$ values were >300 μM, data not shown).

*Sensitivity to Phosphatases.* The carbon–phosphorus bond of Pmp is both chemically and enzymatically stable. We have previously shown that Pmp is resistant to cellular phosphatases (Domchek et al., 1992). In the current study we verify that $F_2$Pmp is stable toward immobilized alkaline phosphatase, as well. Neither HPLC elution characteristics (data not shown) nor SH2 domain binding capacity was altered by exhaustive treatment of a radiolabeled difluorophosphonopeptide with

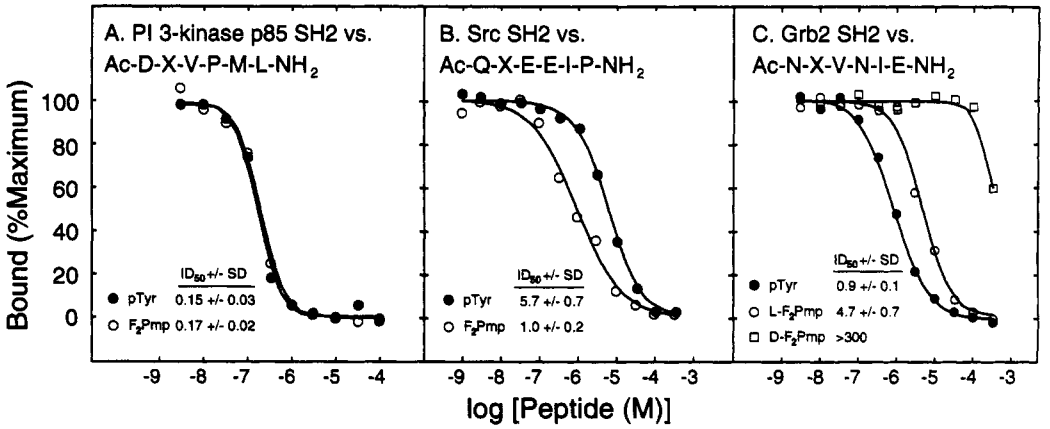


FIGURE 3: Peptide binding with p85, Src, and Grb2 SH2 domains. (A) Binding assays between PDGF receptor-derived peptide sequences and the C-terminal SH2 domain of p85. The peptide (insert) corresponds to the sequence surrounding Tyr751 of the PDGF receptor kinase insert. (B) Binding assays between the Src SH2 domain and peptides corresponding to the sequence surrounding Tyr324 of the polyoma virus middle T antigen which infects hamsters. (C) Binding assays between the Grb2 SH2 domain and peptides corresponding to sequences surrounding IRS-1 Tyr895. Binding assays were conducted as described under Experimental Procedures with the phosphopeptides (X = phosphotyrosine, ●), difluorophosphonopeptides (X = L-$F_2$Pmp, ○) or D-configured $F_2$Pmp (□). In this series all peptides were acetylated and amidated at the N- and C-termini, respectively.

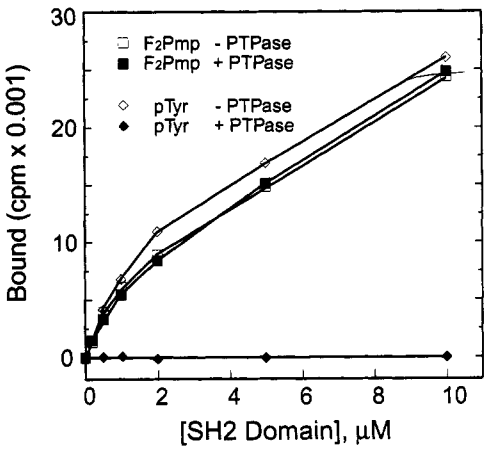


FIGURE 4: Effect of treating peptides with alkaline phosphatases. Peptides Gly-pTyr-Val-Pro-Met-Leu (■, □) and Gly-$F_2$Pmp-Val-Pro-Met-Leu (♦, ◇) were labeled with [$^{125}$I]Bolton-Hunter reagent and purified by HPLC. The radiolabeled peptides were incubated with (■, ♦) or without (□, ◇) alkaline phosphatase–agarose (Sigma) overnight at room temperature. Supernatant radiolabeled peptide solutions were removed and incubated with the indicated concentrations of GST/C-terminal p85 SH2 domain fusion protein and glutathione–agarose. Following an overnight incubation the bound peptides were separated by centrifugation.

the phosphatase (Figure 4). In contrast, similar treatment of the corresponding phosphopeptide resulted in altered HPLC retention and complete loss of SH2 domain binding.

## DISCUSSION

While PTK activity was first observed in association with oncogenic retrovirus proteins, it was subsequently found that these transforming genes actually represent expropriated normal cellular genes (exemplified by v-Src vs c-Src and v-Abl vs c-Abl relationships). We now know that tyrosine phosphorylation represents a major regulatory mechanism used in normal cellular signal transduction pathways [for reviews, see Cantley et al. (1991), Pawson and Schlessinger (1993), and Panayotou and Waterfield (1993)]. Intracellular tyrosines are phosphorylated when surface receptors are engaged by such diverse ligands as growth factors, hormones, or peptide–MHC complexes. Thus, PTK-linked (e.g., PDGF, insulin, and T-cell) receptors generate signals which alter cellular differentiation, growth rate, metabolism, and morphology.

In addition to normal cellular functions, pathologic consequences can frequently be linked to PTK signaling gone awry. As noted above, avian and rodent retroviruses appear to have usurped the genes encoding c-*src* and c-*abl*. Overproduction either of a ligand (the v-*sis* oncogene product) or of a PTK receptor (c-*neu* in breast and ovarian cancer) can also lead to transformed phenotypes. In addition, various mutations activate PTK receptors, exemplified by the truncation of ligand-binding domains (e.g., v-*erb*B and v-*kit*) and point mutations in extracellular (e.g., v-*fms*) or transmembrane (e.g., *neu*/*erb*B2) domains. Chromosomal translocations can also result in oncoproteins having PTK activity, exemplified by BCR-Abl chimera produced in Philadelphia chromosome-positive forms of leukemia. The availability of selective inhibitors of PTK signaling pathways would be useful in studying normal signaling as well as having potential for therapeutic intervention (Burke, 1992).

As the roles which PTKs serve in cytokine-, growth factor-, insulin-, and antigen-mediated signaling become increasingly defined, a common emergent theme has been the induction of protein–protein associations following tyrosine phosphorylation. These high-affinity associations which occur between phosphotyrosyl proteins and the SH2 domains of cytoplasmic signal-transducing molecules are required for signal transduction. Moreover, it is often the case that a given phosphoprotein is able to interact with multiple SH2 proteins. For example, the PDGF receptor interacts with SH2 proteins PI 3-kinase, Src, Ras GAP, SH-PTP2/Syp, and PLC-$\gamma$. The insulin receptor substrate IRS-1 engages an overlapping but distinct collection of SH2 proteins, including PI 3-kinase, SH-PTP2/Syp, Grb2, and Fyn. The multiplicity of potential signaling pathways engaged by each phosphoprotein raises the interesting possibility that a single pathway might be inhibited in isolation. However, different phosphoproteins frequently engage each SH2 protein, suggesting that some of these signaling pathways may be convergent. In either event, SH2 domains provide an attractive target for modulating PTK functions.

Notably, small peptides corresponding to the phosphotyrosyl protein sequences inhibit SH2 domain binding by competing with the phosphoprotein for SH2 domain occupancy [e.g., Escobedo et al. (1991), Fantl et al. (1992), and Auger et al. (1992)], and in certain cases SH2 domain occupancy has the additional consequence of stimulating catalytic activity. For

example, PI 3-kinase (a glycolipid kinase) and SH-PTP2/Syp (a phosphotyrosyl phosphatase) are both activated by phosphoprotein or phosphopeptide interaction with their SH2 domains (Carpenter et al., 1993; Backer et al., 1993; Lechleider et al., 1993; Sugimoto et al., 1994). Therefore, it is difficult to predict *a priori* whether introduction of a phosphopeptide into cells would competitively inhibit or activate a given signaling pathway.

We are interested in these questions, and phosphopeptides provide a useful starting point in the design of SH2 domain inhibitors and/or antagonists. However, phosphopeptides are rapidly dephosphorylated by cellular phosphatases, and thereby inactivated. We previously showed that the phosphonate Pmp is an effective yet less potent mimetic of pTyr which is stable toward phosphorolysis (Domchek et al., 1992). To recover binding affinity lost by replacement of the phosphate ester oxygen with the phosphonate methylene, we developed synthetic reagents for the incorporation of modified Pmp derivatives in peptides by solid-phase synthetic methods (Burke et al., 1993a,b; Smyth & Burke, 1994). These analogues contain fluorine substituents at the methylene bridge which increase phosphonate acidity and the potential for hydrogen bonding.

Results indicate that L-$F_2$Pmp in particular is a valuable, new analogue for the preparation of phosphatase-resistant SH2 domain inhibitory peptides. In several cases pTyr was replaced by $F_2$Pmp in phosphopeptides known to bind to SH2 domains with high affinity and selectivity. $F_2$Pmp and pTyr peptides bearing identical pTyr-Met/Val-Xxx-Met motifs bind to the PI 3-kinase p85 C–SH2 domain with equal affinity (Figures 1 and 3A). Moreover, an $F_2$Pmp peptide having a pTyr-Glu-Glu-Ile sequence (Eck et al., 1993; Payne et al., 1993; Songyang et al., 1993; Waksman et al., 1993) binds to the Src SH2 domain with 5-fold higher affinity than the corresponding pTyr peptide, whereas $F_2$Pmp substitution in a peptide modeled on the Grb2 SH2 domain binding site of IRS-1 reduces affinity 5-fold. The potencies of $F_2$Pmp sequences relative to the corresponding pTyr peptides may vary among classes of SH2 domains, depending on subtle differences in the structures of peptide–SH2 domain complexes. Work is in progress to explore the use of $F_2$Pmp peptides as inhibitors of SH2 domain-mediated events in cells to learn more about these signaling pathways. These studies should demonstrate the feasibility of using SH2 domains as targets for *in vivo* inhibition in such disorders as cancer and diabetes, and when immunologic modulation might be desirable.

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