Accelerated Publications

Inhibition of SH2 Domain/Phosphoprotein Association by a Nonhydrolyzable Phosphonopeptide†

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Received July 6, 1992; Revised Manuscript Received August 14, 1992

ABSTRACT: Using the association between the pp60$^{c\text{-}src}$/polyoma virus middle T antigen (mT) complex and phosphatidylinositol 3′-kinase (PI 3-kinase) as a prototype for phosphoprotein–SH2 domain interactions, we tested whether a nonhydrolyzable phosphonopeptide would inhibit association. (Phosphonomethyl)-phenylalanine (Pmp) is a nonnatural analogue of phosphotyrosine in which the >C–O–PO_3H_2 moiety is replaced by >C–CH_2–PO_3H_2. We synthesized a 13 amino acid phosphonopeptide (mT-Pmp315), a related phosphopeptide (mT-pY315), and an unmodified sequence (mT-Y315), all corresponding to the pp60$^{c\text{-}src}$-phosphorylated site of the mT which is within a YMXM motif common to proteins that bind to and activate PI 3-kinase. Only the phosphonopeptide persistently blocked the in vitro association of the baculovirus-expressed pp60$^{c\text{-}src}$/mT complex with cytosolic PI 3-kinase activity. Sustained inhibition of association by the phosphopeptide required the additional presence of vanadate, a potent protein tyrosine phosphatase (PTPase) inhibitor. The phosphopeptide and L-phosphonopeptide bound tightly ($K_D \approx 10$–20 nM) and specifically to isolated SH2 domains of PI 3-kinase p85, demonstrating that the mechanism of inhibited association is competitive binding to PI 3-kinase SH2 domains. We conclude that the appropriate phosphonopeptide sequence inhibits the interaction between a tyrosine-phosphorylated protein and a cognate SH2 domain-containing protein and is resistant to the actions of PTPases. Proteolytically stable phosphonopeptide derivatives should be useful inhibitors of protein–protein interactions when introduced into cells and may provide a basis for the rational design of a new class of chemotherapeutic agent.

Activation of receptor and nonreceptor tyrosine kinases is accompanied by phosphorylation of specific tyrosine residues within the kinases themselves (autophosphorylation) as well as on additional proteins (substrate phosphorylation) (Ullrich & Schlessinger, 1990). Recent evidence suggests that selected sequences surrounding tyrosine phosphorylation sites are recognized by additional cytoplasmic proteins containing *src*-homology 2 (SH2)[1] domains (Koch et al., 1991; Cantley et al., 1991), including such proteins as pp60$^{c\text{-}src}$ itself, phospholipase Cγ (PLCγ), GTPase activating protein (GAP) associated with *ras*, and phosphatidylinositol 3′-kinase (PI 3-kinase). Although the functional significance of these interactions is not fully understood, their ubiquity suggests an important role in intracellular signal transduction.

Phosphoprotein recognition by SH2 domain-containing proteins is thought to derive its specificity from phosphorylation per se and from the surrounding amino acid sequence. A recognition motif for the p85 subunit of PI 3-kinase, probably the best characterized, is Tyr-Met/Val-Xxx-Met (YMXM or YVXM). Proteins that interact with PI 3-kinase frequently contain these motifs, including the PDGF, CSF-1 (c-*fms*), and *kit*-encoded receptors, the pp60$^{c\text{-}src}$-associated polyoma virus middle T antigen (mT), and the insulin receptor substrate IRS-1 (Kazlauskas & Cooper, 1989; Cantley et al., 1991; Sun et al., 1991; Reedijk et al., 1992; Lev et al., 1992). The observation of Escobedo et al. (1991) that relatively short peptide sequences corresponding to PDGF receptor phosphorylation sites inhibited the interaction between the activated PDGF receptor and PI 3-kinase opened the way for the current study. In this and subsequent studies 5-12 amino acid phosphopeptides corresponding to YMXM or YVXM sequences surrounding Tyr740 or Tyr751 of the human PDGFβ receptor, Tyr315 of mouse mT or Tyr298 of hamster mT, or any of the six Tyr residues of rat IRS-1 have been shown to block association of PI 3-kinase activity with the PDGF receptor (Escobedo et al., 1991; Fantl et al., 1992), pp60$^{c\text{-}src}$/mT complex (Auger et al., 1992; Carpenter et al., submitted), and IRS-1 (Backer et al., 1992; Myers et al., 1992),

† Supported in part by a Career Development Award from the Juvenile Diabetes Foundation, International, by grants to S.E.S. from the NIH (DK08366) and NSF (DMB90-04670), and by an NIH Diabetes and Endocrinology Research Center Grant to the Joslin Diabetes Center.

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[1] Abbreviations: BOP, (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate; CSF-1R (c-*fms*), colony-stimulating factor receptor; Fmoc-Pmp(tBu)$_2$-OH, racemic *N*-Fmoc-*p*-[(di-*tert*-butylphosphono)methyl]phenylalanine; Fmoc-Tyr(OP(OCH$_3$)$_2$), *N*-Fmoc-*O*-(*O*,*O*-dimethoxyphosphoryl)-L-tyrosine; GAP, GTPase activating protein of *ras*; HOBt, 1-hydroxybenzotriazole; IRS-1, insulin receptor substrate 1; mT, polyoma virus middle T antigen; PDGF, platelet-derived growth factor; PI 3-kinase, phosphatidylinositol 3′-kinase; PLCγ, phospholipase Cγ; Pmp, (phosphonomethyl)phenylalanine; PTPase, protein tyrosine phosphatase; SH2, *src*-homology 2.

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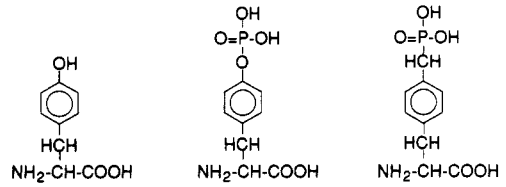
A. H-Glu-Glu-Glu-Xxx-Met-Pro-Met-Glu-Asp-Leu-Tyr-OH



B. Tyr C. pTyr D. Pmp

FIGURE 1: Peptide and amino acid structures. (A) Sequence of peptides corresponding to the region of the mouse polyoma middle T antigen surrounding Tyr315. For the three peptides used in these studies Xxx is either (B) tyrosine (Tyr), (C) phosphotyrosine (pTyr), or (D) (phosphonomethyl)phenylalanine (Pmp).

respectively.

These findings suggest that a linear sequence surrounding a phosphorylated tyrosine is sufficient for SH2 domain recognition and that an extended three-dimensional structure surrounding the targeted site contributes relatively little to the interaction. To analyze the mechanism of phosphoprotein/SH2 domain association further, we recently found that these and additional peptides bind specifically and with high affinity directly to the isolated SH2 domains of PI 3-kinase (Felder et al., submitted; Piccione et al., manuscript in preparation). Therefore, peptides containing phosphotyrosine within the correct sequence motifs (YMXM or YVXM) bind to the SH2 domains of PI 3-kinase p85 and act as competitive inhibitors of p85 association with the phosphoprotein.

We reasoned that by incorporating a nonhydrolyzable analogue of phosphotyrosine within the same peptide sequences we might generate a new class of peptides which would retain the capacity to bind to p85 SH2 domains and, in addition, be resistant to the actions of cellular PTPases. (Phosphonomethyl)phenylalanine (Pmp) is a phosphotyrosine congener in which the $>C-O-PO_3H_2$ group of phosphotyrosine is replaced by $>C-CH_2-PO_3H_2$. In this study three peptides having the same linear sequence corresponding to residues surrounding Tyr315 of the mouse polyoma virus middle T antigen (mT) were synthesized (Figure 1A). The peptides differ at the para position of the targeted tyrosine, having a free hydroxyl, a phosphate, or the nonhydrolyzable, methylene-bridged phosphonate (Figure 1B–D). Both the phosphopeptide and the phosphonopeptide were found to bind to isolated SH2 domains of the p85 subunit of PI 3-kinase. However, in the absence of PTPase inhibitors only the phosphonopeptide inhibited pp60$^{c\text{-}src}$/mT complex association with cytosolic PI 3-kinase activity.

EXPERIMENTAL PROCEDURES

Peptide Synthesis. Syntheses of peptides E-E-E-X-M-P-M-E-D-L-Y, where X = Pmp, phosphotyrosine, or tyrosine, were conducted on a Milligen/Biosearch 9600 synthesizer using 4-alkoxybenzyl alcohol–polystyrene resin (PAC, Milligen/Biosearch). The N^{α}-Fmoc protecting group was used throughout in conjunction with the *tert*-butyl group side-chain protection of Asp, Glu, and Tyr. Racemic *N*-Fmoc-*p*-[(di-*tert*-butylphosphono)methyl]phenylalanine [Fmoc-Pmp(tBu)$_2$-OH] (Shoelson et al., 1991; Burke et al., 1991) and *N*-Fmoc-*O*-(*O*,*O*-dimethoxyphosphoryl)-L-tyrosine [Fmoc-Tyr-(OP(OCH$_3$)$_2$)] (Kitas et al., 1991) were used as protected synthons for incorporation of Pmp and phosphotyrosine, respectively. Peptide bond-forming reactions with 0.2 M N^{α}-Fmoc-amino acid, 1-hydroxybenzotriazole (HOBt), and (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) were conducted for 1 h prior to incorporation of Fmoc-Pmp(tBu)$_2$-OH or Fmoc-Tyr(OP-(OCH$_3$)$_2$) and for 4 h afterward. Fmoc-Pmp(tBu)$_2$-OH and Fmoc-Tyr(OP(OCH$_3$)$_2$) were coupled manually with a 2-fold excess of Fmoc-amino acid, HOBT, and BOP. Peptides were cleaved from the resin, and side-chain protecting groups were simultaneously removed by treatment with trifluoroacetic acid, thioanisole, ethanedithiol, and anisole (90:5:3:2) for 2 h at 22 °C. Methyl protecting groups on phosphotyrosine were removed during a second stage of deprotection with trimethylsilyl bromide (Kitas et al., 1991). All peptides were precipitated with diethyl ether (4 °C) and desalted on a column of Bio-Gel P2. Peptides were further purified, and D- and L-forms of the Pmp peptides were separated using a Waters Prep 4000 HPLC equipped with a Dynamax-300A 12-μm C8 column (41.4 × 250 mm). Amino acid analyses (ABI 420) and results obtained from plasma desorption mass spectrometry (ABI BioIon) were as expected (Shoelson et al., 1991). Peptides containing D- or L-Pmp were differentiated by amino acid analysis using *N*-isobutyryl-L-cysteine derivatives (Bruckner et al., 1989, 1991). As noted for the common natural amino acids, the isobutyryl-L-Cys/L-Pmp enantiomer eluted from the reversed-phase HPLC earlier than the corresponding *N*-isobutyryl-L-Cys/D-Pmp derivative.

Inhibiting in Vitro Association of PI 3-Kinase Activity with Immunopurified pp60$^{c\text{-}src}$/mT. Baculovirus-expressed pp60$^{c\text{-}src}$/mT complexes from infected Sf9 insect cell (Piwnica-Worms et al., 1990) lysates were immunopurified as described (Auger et al., 1990, 1992). PI 3-kinase activity was obtained directly from crude Balb/3T3 cell lysates as follows. After confluent 10-cm dishes were washed with phosphate-buffered saline, Balb/3T3 cells were treated with lysis buffer (137 mM NaCl, 20 mM Hepes, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 1% Nonidet P-40, and 1 μg/mL each of leupeptin, pepstatin A, and aprotinin, pH 7.25) in the presence or absence of 0.15 mM sodium vanadate. Following a 10-min incubation with lysis buffer, cells were scape-harvested, and particulate debris was removed from the lysates by centrifugation at 12000*g* for 5 min at 4 °C. Varying concentrations of peptides were added directly to the lysates, and mixtures were incubated at 4 °C for 30 min.

Anti-mT antibodies (2 h, 4 °C) and protein A–Sepharose beads (30 min, 4 °C) were sequentially added to the lysate–peptide mixtures to immunoprecipitate pp60$^{c\text{-}src}$/mT/PI 3-kinase complexes. After being washed, the beads were used to catalyze phosphatidylinositol phosphorylation as described (Whitman et al., 1985; Auger et al., 1989, 1990). Sonicated phospholipids were added to the beads, and phosphorylation reactions were initiated with 50 μM [γ-^{32}P]ATP and 5 mM $MgCl_2$ in 20 mM HEPES, pH 7.5. Following 5-min incubations at 22 °C the 50-μL reactions were terminated by addition of 80 μL of 1 N HCl and 1 μL of 0.5 mM ethylenediaminetetraacetic acid. Lipids were extracted with 0.16 mL of methanol–chloroform (1:1) and separated by thin-layer chromatography (Auger et al., 1990).

Peptide Binding to Isolated Glutathione S-Transferase/SH2 Domain Fusion Proteins. N-Terminal p85 SH2/GST fusion proteins (100 nM) (Yoakim et al., 1992), 35 fmol of HPLC-purified, [^{125}I]-Bolton–Hunter reagent-treated phosphopeptide (67 nCi), and varying concentrations of unlabeled peptides were combined in a 200-μL total volume of 20 mM Tris-HCl, 250 mM NaCl, 0.1% bovine serum albumin, and 10 mM dithiothreitol, pH 7.4. Glutathione–agarose (25 μL of a 1:4 aqueous slurry; Molecular Probes) was added, and the samples were incubated at 22 °C for 4 h. Following

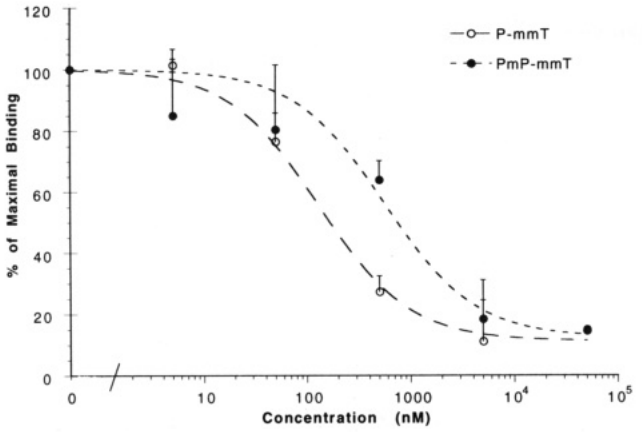


FIGURE 2: Concentration dependencies for inhibition of the association between the baculovirus-expressed mT/pp60^{src} complex and cytosolic PI 3-kinase activity by phosphopeptide mT-pY315 (open circles) and racemic phosphonopeptide mT-Pmp315 (closed circles). Peptides were incubated for 30 min with cytosol from Balb/3T3 cells in the presence of 200 μM sodium vanadate and combined with the baculovirus-expressed mT/pp60^{src} complex, anti-mT antibodies (2 h), and protein A–Sepharose (30 min). Precipitated protein complexes were used to catalyze phosphorylation of phosphatidylinositol as described under Experimental Procedures.

centrifugation for 5 min at 12000g, supernatant solutions were removed by aspiration, and [^{125}I] radioactivity associated with the pellets was determined with a γ-counter. Additional details of the phosphopeptide/SH2 binding assay and N-terminal p85 SH2 domain specificity are being submitted separately (Piccione et al., manuscript in preparation).

Amino Acid Side-Chain pK_a. (Phosphonomethyl)phenylalanine and phosphotyrosine were suspended in water and brought into solution by addition of sodium hydroxide to yield a 0.2 M solution, pH >10.3. Aliquots (10 μL) of 6 N HCl were added with mixing, and the pH was recorded.

RESULTS

Inhibition of PI 3-Kinase Association with the pp60$^{c\text{-}src}$/mT Complex. Cytosolic PI 3-kinase activity associates with pp60$^{c\text{-}src}$/mT complexes and can be immunoprecipitated with antibodies against either pp60$^{c\text{-}src}$ or mT (Whitman et al., 1985, 1988; Kaplan et al., 1986). This association occurs only if the pp60$^{c\text{-}src}$ is an active protein tyrosine kinase, suggesting that a key feature of the recognition involves tyrosine phosphorylation. Furthermore, the association can be blocked by a phosphopeptide corresponding to the sequence surrounding Tyr298 of hamster polyoma mT (Auger et al., 1992). In the current study we prepared a phosphopeptide and matched nonhydrolyzable phosphonopeptide corresponding to the sequence surrounding Tyr315 of mouse polyoma mT (Figure 1). In the presence of 200 μM sodium vanadate both peptides inhibited association between pp60$^{c\text{-}src}$/mT and PI 3-kinase, with ID_{50} values of $\approx$100 and $\approx$800 nM for the phosphopeptide and racemic phosphonopeptide, respectively (Figure 2). An unphosphorylated peptide having the same hamster mT sequence had no detectable activity under these conditions (Auger et al., 1992).

Similar experiments were conducted in the absence of sodium vanadate to determine whether inhibition would persist in the presence of active protein tyrosine phosphatases (PTPases). Concentrations of peptides were chosen to give $\approx$50% inhibition of association between pp60$^{c\text{-}src}$/mT and PI 3-kinase activity (Figure 3). Phosphopeptide-mediated inhibition of association was abolished in the absence of vanadate due to tyrosine dephosphorylation. By contrast, inhibition by

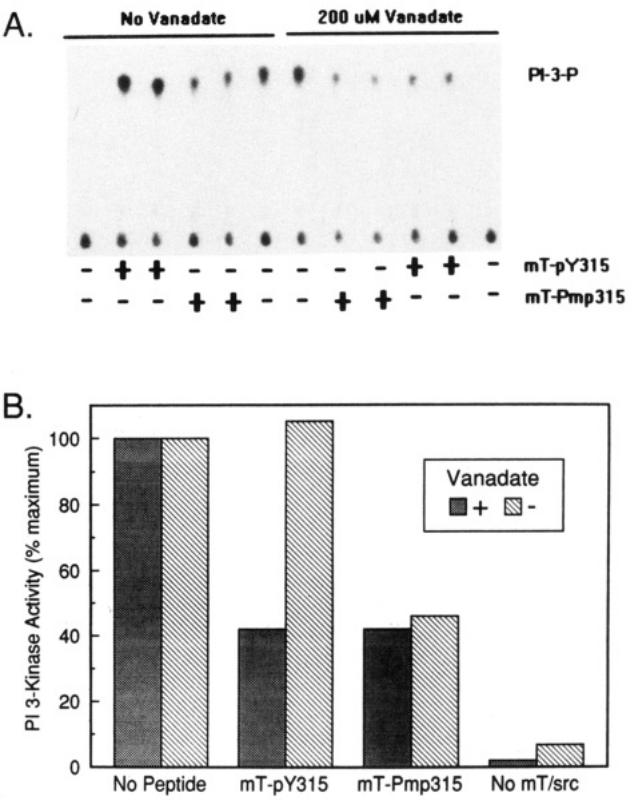


FIGURE 3: Phosphopeptide vs phosphonopeptide inhibition of association between the baculovirus-expressed mT/pp60^{src} complex and cytosolic PI 3-kinase activity in the presence and absence of sodium vanadate. Panel A is an autoradiogram of one experiment; the upper spot is the product, [^{32}P]phosphatidylinositol 3-phosphate (PI-3-P), and the lower spot is the origin. The outermost two control lanes show the amount of enzyme activity observed in the absence of the added mT/pp60^{src} complex; all other lanes were in the presence of the added mT/pp60^{src} complex. The innermost two control lanes show total activity in the absence of peptide; the effects of added phosphopeptide (mT-pY315) or phosphonopeptide (mT-Pmp315) are seen in the indicated lanes in the absence (left) or presence (right) of sodium vanadate. Each lane represents a separate assay. Panel B represents the averages of data from two such experiments, each containing two separate assays (n = 4). Method: Peptides were incubated first with cytosol from Balb/3T3 cells at 4 °C for 30 min the presence and absence of 200 μM sodium vanadate; peptide concentrations were chosen to inhibit $\approx$one-half of associated PI 3-kinase activity (Figure 2, 75–100 nM mT-pY315, 0.75–1.0 μM mT-Pmp315). These mixtures were combined with the baculovirus-expressed mT/pp60^{src} complex, anti-mT antibodies (2 h), and protein A–Sepharose (30 min). Following washes, precipitated complexes were used to catalyze phosphorylation of phosphatidylinositol as described under Experimental Procedures.

the phosphonopeptide persisted even in the absence of PTPase inhibition, which demonstrates the inability of PTPases to hydrolyze the C–P bond of Pmp. In fact, Pmp is unaltered by prolonged incubation with high concentrations of expressed PTPases (Chatterjee et al., 1992) and is stable under much harsher hydrolytic conditions (e.g., boiling 6.0 N HCl for 18 h; Marseigne & Roques, 1988).

Phosphopeptide and Phosphonopeptide Binding to p85 SH2 Domains. To investigate the mechanism of phosphonopeptide inhibition of association, we analyzed direct binding to an isolated SH2 domain of the p85 of PI 3-kinase. In the assay, tracer amounts of [^{125}I]-labeled phosphopeptide, SH2 domain/GST fusion protein, and varying concentrations of unlabeled peptides were incubated together along with glutathione–agarose. In the absence of unlabeled phosphopeptide 10–30% of total added radioactivity was precipitated specifically with the glutathione beads; in the presence of excess phosphopeptide or in the absence of SH2/GST fusion protein 2–4%

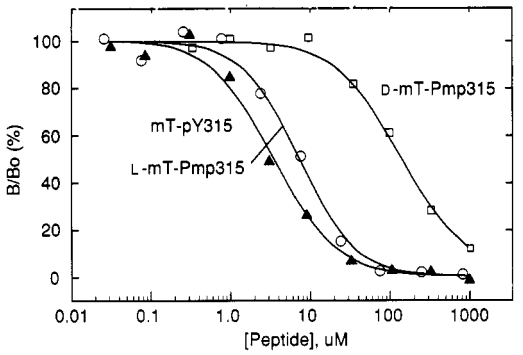


FIGURE 4: Competition of binding between peptides and p85 SH2/GST fusion protein. N-Terminal p85 SH2/GST fusion protein, [^{125}I]-labeled phosphopeptide, and varying concentrations of unlabeled peptides were combined in a 200-μL total volume of 20 mM Tris-HCl, 250 mM NaCl, 0.1% bovine serum albumin, and 10 mM dithiothreitol, pH 7.4. Glutathione–agarose (25 μL of a 1:8 aqueous slurry) was added, and the samples were incubated at 22 °C for 4 h. Following centrifugation for 5 min at 12000g, supernatant solutions were removed by aspiration, and [^{125}I] radioactivity associated with the pellets was determined with a γ-counter.

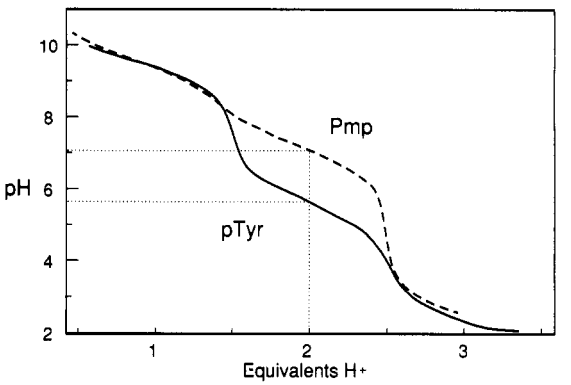


FIGURE 5: Determination of side-chain pK_a values for phosphotyrosine and (phosphonomethyl)phenylalanine. Free amino acids were dissolved in aqueous sodium hydroxide, pH > 10; aliquots of HCl were added and the pH was recorded.

of total radioactivity was found to associate (nonspecifically) with the glutathione beads. ID_{50} values were determined as the concentration of unlabeled peptide required to inhibit 50% of specific [^{125}I]-labeled phosphopeptide binding. Binding data were best fit to the sigmoid dose–response equation $y = [a/(1 + e^{x-c})] + d$ as described (DeLean et al., 1978). A calculated ID_{50} value for the phosphopeptide mT-pY315 was 3.6 μM (Figure 4). Different affinities were observed for the two isomers of the phosphonopeptide: ID_{50} values for the L- and D-isomers were 7.2 and 131 μM, respectively. Therefore, L-mT-Pmp315 binds to the N-terminal SH2 domain of p85 2-fold weaker than the corresponding phosphopeptide (which contains L-phosphotyrosine), whereas D-mT-Pmp315 binds to the N-terminal SH2 of p85 much more weakly with a relative ID_{50} value $\approx$30–40-fold higher than that of the phosphopeptide. As the D- and L-forms of the phosphonopeptide were separated by HPLC and eluted closely together (Shoelson et al., 1991), traces of the opposite isomer might contaminate the HPLC-separated isomers. Therefore, D-mT-Pmp315 could bind with even weaker relative affinity. The nonphosphorylated peptide mT-Y315 exhibited no binding.

Whereas relative affinities can be obtained from the competition assay, absolute values for K_D are not obtained due, in part, to the high concentration of SH2 domain/GST fusion protein compared to K_D (Piccione et al., manuscript in preparation). Therefore, we analyzed mT-pY315 binding to p85 SH2 domains using biospecific interaction analysis as previously described (Felder et al., submitted) to obtain a better estimate of K_D. Series of data were obtained during real-time binding experiments with different concentrations of SH2/GST fusion protein (data not shown). The data, extrapolated to infinite time to estimate values for "steady-state" binding, were analyzed as bound/free vs bound analogous to a Scatchard plot to obtain an estimated K_D of 10 nM. By comparison, estimated K_D values for L- and D-mT-Pmp315 are 20 nM and $\geq$0.3–0.4 μM, respectively.

Phosphonate vs Phosphate pK_a. To assess possible reasons for reduced affinity of the phosphonopeptide vs the phosphopeptide, we determined side-chain pK_a values. For phosphotyrosine pK_3 ($-OPO_3^{2-} \leftrightarrow -OPO_3H^-$) was 5.7, in close agreement with a reported value (Cooper et al., 1983). The corresponding pK_3 value for Pmp was 7.1 (Figure 5). Therefore, at neutral pH the phosphate side chain carries a –2 charge whereas half the phosphonate side chains have a –2 charge and the other half (being protonated) have a charge of –1. As a –2 charge on phosphotyrosine (or Pmp) might be necessary for high-affinity interaction with basic residues within the SH2 domain (Koch et al., 1991; Mayer et al., 1992; Williams et al., manuscript in preparation), different side-chain pK_a values might explain the different binding affinities observed for the phosphopeptide vs the L-phosphonopeptide.

DISCUSSION

Transformation of fibroblasts by polyoma virus requires the presence of middle T antigen, which is a substrate of cellular tyrosine kinases and regulates tyrosine kinase activity. A small percentage of mT present in cells associates with such protooncogenes as pp60$^{c\text{-}src}$, pp62$^{c\text{-}yes}$, and pp59^{fyn} (Cheng et al., 1988; Courtneige et al., 1983; Kornbluth et al., 1987; Kypta et al., 1988) and, for pp60$^{c\text{-}src}$, activates protein tyrosine kinase activity. Furthermore, phosphorylation of mT at Tyr315 is crucial for efficient transformation, tumorigenesis, and association with PI 3-kinase (Carmichael et al., 1984; Whitman et al., 1985; Kaplan et al., 1986, 1987; Talmage et al., 1989). Notably, the PI 3-kinase activity that associates with the pp60$^{c\text{-}src}$/mT complex also associates with stimulated (phosphorylated) growth factor receptors (Kazlauskas & Cooper, 1989; Ruderman et al., 1990; Cantley et al., 1991; Hu, et al., 1992; Lev et al., 1992; Reedjiket al., 1992) and their tyrosine-phosphorylated substrates, exemplified by IRS-1 (Sun et al., 1991; Shoelson et al., 1982; Lavan et al., 1992; Backer et al., 1992). Though physiological roles for the phosphatidylinositol 3-phosphate products of PI 3-kinase have yet to be determined, relationships between protooncogene and oncogene tyrosine kinases and PI 3-kinase suggest an important role in both regulated and deregulated cellular growth.

The mechanism of phosphoprotein/PI 3-kinase association is thought to involve direct interactions between *src*–homology 2 (SH2) domains of the p85 subunit of PI 3-kinase and phosphorylated sequences within the viral transforming protein (mT), the growth factor receptors, or their substrates (IRS-1). Therefore, it is particularly noteworthy that many of the phosphoproteins that are recognized by PI 3-kinase contain tyrosine residues within Tyr-Met-Xxx-Met (YMXM) or Tyr-Val-XXX-Met (YVXM) motifs, and in each case tyrosine phosphorylation appears to be necessary for the interaction. In support of this, Tyr → Phe substitutions (within the YM/VXM motifs) dramatically decrease association (Kaplan et al., 1986; Courtneidge, & Heber, 1987; Kazlauskas & Cooper, 1989). In addition, phosphoprotein/PI 3-kinase association is blocked either by free p85 SH2 domains or by phospho-

peptides corresponding to the sequences surrounding YMXM or YVXM sequences.

The ability of phosphopeptides to both potently inhibit in vitro phosphoprotein/SH2 domain interactions and directly stimulate PI 3-kinase enzymatic activity (Backer et al., 1992; Carpenter et al., submitted) prompted the current study. We would like to conduct similar studies in intact cells. In vitro, cytosolic protein tyrosine phosphatases can be inhibited by the addition of PTPase inhibitors, and phosphopeptide dephosphorylation is suppressed. In the absence of PTPase inhibition, phosphopeptide dephosphorylation occurs and phosphopeptide-mediated inhibition of phosphoprotein/PI 3-kinase association is lost, as shown in Figure 3. For studies to be conducted in intact cells (where introduction of PTPase inhibitors would often be lethal and would certainly confound potential observations), we designed analogues related to the active phosphopeptides which are resistant to the actions of PTPases. These analogues are identical to the active phosphoproteins, except phosphotyrosine has been replaced by (phosphonomethyl)phenylalanine (a derivative which has a methylene bridge between phosphorus and the phenyl ring in place of oxygen). We now show that phosphonopeptides, like the related phosphopeptides, inhibit phosphoprotein–SH2 domain interactions in vitro. The peptide phosphonate described here has the desired properties, including ease of synthesis, high-affinity and high-specificity binding to PI 3-kinase SH2 domains, and resistance to the actions of PTPases.

Although the peptides used in this study were based on a sequence surrounding Tyr315 of mouse mT, these peptides are not specific inhibitors of pp60$^{c\text{-}src}$/mT association with PI 3-kinase. Phosphopeptides corresponding to sequences surrounding YMXM or YVXM sites from PDGF, CSF-1, or *kit*-encoded receptors or IRS-1 all bind to p85 SH2 domains with nearly equivalent affinity to the mT-derived peptides (Piccione et al., manuscript in preparation), and many of the same phosphopeptides inhibit association of phosphorylated IRS-1 and PI 3-kinase (Backer et al., 1992). Therefore, introduction of any related phosphonopeptide into cells may inhibit all phosphoprotein interactions with PI 3-kinase. In addition, selected phosphopeptides stimulate PI 3-kinase activity 2–3-fold (Backer et al., 1992; Carpenter et al., submitted). Therefore, observed phenotypes following introduction of such phosphonopeptides into cells may result either from blocked phosphoprotein–PI 3-kinase p85 interactions or from direct stimulatory effects. In either event, these results should provide insights into the physiological role of PI 3-kinase and its phosphorylated products.

Whereas we used the pp60$^{c\text{-}src}$/mT complex interaction with PI 3-kinase as a prototype for phosphoprotein–SH2 domain interactions, these results should readily extrapolate to additional phosphoprotein–SH2 domain interactions. For example, GAP binding to the activated PDGF receptor is inhibited by a synthetic phosphopeptide corresponding to the sequence surrounding PDGF receptor Tyr771 (Fantl et al., 1992), and mutagenesis of PDGF receptor Tyr771 to Phe decreases GAP binding (Kashishian et al., 1992). Similarly, PLCγ interactions with the *flg*-encoded FGF receptor may be through an interaction with Tyr766 (Mohammadi et al., 1991) and with the EGF receptor via Tyr992 (Rotin et al., 1992). Specificity for phosphoprotein binding by these SH2 domain-containing proteins may also depend on tyrosine phosphorylation and a linear sequence. However, for most SH2 proteins recognition motifs are unknown. As more protein/protein complexes are identified and their interactions deciphered, an increasing number of specificity motifs will be identified. All such phosphoprotein–SH2 interactions should be equally amenable to targeted inhibition by phosphopeptides and related phosphonopeptides.

ACKNOWLEDGMENT

The authors sincerely thank Helen Piwnica-Worms and Brian Schaffhausen for kindly supplying the mT- and pp60$^{c\text{-}src}$-baculovirus constructs and the p85 SH2 domain fusion protein, respectively, and Jonathan Backer, Lew Cantley, Steven Felder, Tony Pawson, Josef Schlessinger, and Morris White for helpful discussions.

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