Direct Determination of the Sequence Recognition Requirements of the SH2 Domains of SH-PTP2†

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ABSTRACT: SH-PTP2 is a widely-expressed protein tyrosine phosphatase with two tandem SH2 (*src* homology 2) domains and a C-terminal catalytic domain. Glutathione *S*-transferase fusions of the SH2 domains alone and of a catalytically inactive full-length mutant were made, and binding assays were developed using the purified fusion proteins to directly determine what residues are involved in the recognition of binding targets by the SH2 domains. The binding kinetics of the SH2 domains to a phosphotyrosyl-containing peptide of the sequence surrounding Tyr^{1009} of the platelet-derived growth factor receptor (PDGFR) β subunit [DTSSVL(pY)TAVQPN] were determined by surface plasmon resonance, confirming that this is a high-affinity binding ligand. Using various N- and C-terminal truncations of this peptide as competitors in the binding assays, the minimum peptide that served as a high-affinity binding ligand was found to be VL(pY)TAV. Systematic Ala substitutions of this peptide indicated that in addition to the phosphotyrosine (pY), the critical residues for recognition and binding are at pY+1 and pY+3 as previously reported, and notably at pY−2 as well. Binding competition results with these and other PDGFR, IRP, and IRS-1 peptides suggested some general rules for sequence recognition by the SH2 domains of SH-PTP2. Peptides that bind to the SH2 domains in the binding assays were also found to stimulate the phosphatase activity of SH-PTP2.

Protein tyrosine phosphorylation plays an essential role in regulating cell function. The tyrosine phosphorylation status of a protein in the cell reflects the balance between the combined activities of protein tyrosine kinases (PTKs)[1] and protein tyrosine phosphatases (PTPases). One mode of signal transmission by tyrosine phosphorylation involves SH2 domains. These domains consist of about 100 amino acids and bind to pY residues in proteins, with the binding specificity apparently determined by the amino acids around the phosphotyrosine (Anderson *et al.*, 1990; Cantley *et al.*, 1991; Koch *et al.*, 1991; Pawson & Gish, 1992; Birge & Hanafusa, 1993). SH2 domains are present in a variety of signaling proteins, and the proteins can be broadly divided into two classes. The first class of proteins has no inherent enzymatic activity in the polypeptide chain that contains the SH2 domain (e.g., Grb2, p85 subunit of PI-3 kinase). The second class of proteins is enzymes that have SH2 domains in their structure (e.g., *src*, PLCγ, PTP1C, SH-PTP2). The PTPases in this latter class are unique in that by definition they have two different phosphotyrosine-binding motifs, the catalytic domain and the SH2 domain.

The crystal structures of *src* and *lck* SH2 domains with binding peptides revealed that the major sites of interaction with their pY peptide targets are at the pY residue and a hydrophobic residue three positions C-terminal to pY (pY+3), as well as with the residue at pY+1 (Waksman *et al.*, 1992, 1993; Eck *et al.*, 1993, 1994). The peptide fits into the SH2 domain like a two-pronged plug fitting into a socket. Recently the structure of the PLCγ SH2 domain has been solved (Pascal *et al.*, 1994). This SH2 domain has a hydrophobic peptide binding groove, and contacts are made with the pY peptide target at the same residues described for the *src* SH2 domain (pY, pY+1, and pY+3) as well as residues outside this region, especially pY+4 to pY+6. These data suggest that the PLCγ SH2 domain may define another class of SH2 domain. Binding specificity data using peptide library screens are also consistent with different subtypes of SH2 domains (Songyang *et al.*, 1993).

Recently we and others have cloned a widely expressed SH2 domain-containing tyrosine phosphatase known as SH-PTP2 (Bastien *et al.*, 1993; Freeman *et al.*, 1992) and also referred to as PTP2C (Ahmad *et al.*, 1993), SH-PTP3 (Adachi *et al.*, 1992), Syp (Feng *et al.*, 1993), and PTP1D (Vogel *et al.*, 1993). It has been shown that this protein associates through its SH2 domains with PDGFR β subunit at pY residue 1009 (Lechleider *et al.*, 1993a). In this paper we have studied the binding of the SH2 domains alone, as

† This paper is dedicated to the memory of Sandra Huyer (1935−1992).

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[1] Abbreviations: PTK, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; SH2, *src* homology 2; pY, phosphotyrosyl; PLC, phospholipase C; PDGFR, platelet-derived growth factor receptor β; GT, glutathione; GST, glutathione *S*-transferase; GST−SH2, fusion of GST with the SH2 domains of SH-PTP2; GST−SH-PTP2, fusion of GST with SH-PTP2; GST−SH-PTP2-C→S, fusion of GST with the catalytically-inactive SH-PTP2 (Cys^{459}→Ser); GST−PTP2-C→S, fusion of GST with the mutated catalytic domain of SH-PTP2 (without the SH2 domains); SH-PTP2-C→S, thrombin-cleaved form of the GST fusion; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; BAEE, N^{α}-benzoyl-L-arginine ethyl ester; IRS-1, insulin receptor substrate-1; IRP, insulin receptor protein; RCM lysozyme, reduced, carboxyamidomethylated and maleyated lysozyme.

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well as a catalytically inactive full-length SH-PTP2, to an immobilized 13-mer peptide of the PDGFR containing the pY residue 1009. A competitive binding assay was also developed for measuring the relative affinities of pY peptides to the SH2 domains. The results from this study show that two amino acids N-terminal, and three amino acids C-terminal, to the phosphotyrosine are essential for high-affinity binding, with the major interactions at pY+1, pY+3, and pY−2, in addition to the phosphotyrosine. Furthermore, the minimal peptide that binds with high affinity to the SH2 domains also stimulates the phosphatase activity of the enzyme.

EXPERIMENTAL PROCEDURES

Materials. All chemicals used were of reagent grade from Sigma. Triton X-100, biotin, and streptavidin were from Pierce. Anti-phosphotyrosine antibody was from UBI. GT-Sepharose was purchased from Pharmacia as a suspension in 20% ethanol, and GT-agarose was from Sigma. [γ-^{32}P]-ATP was from NEN, and Aquasol scintillation fluid was from DuPont. Protein determination was by the method of Bradford (1976), using a kit from Bio-Rad.

Preparation of pY Peptides. The pY peptides were synthesized and purified by California Peptide Research Inc. (Napa, CA). The phosphopeptides with free carboxyl terminals were synthesized using Boc-amino acids while the carboxy amide peptides were synthesized with Fmoc-amino acids. Fmoc dimethyl phosphotyrosine derivative was used for both syntheses. Mass spectral analysis and amino acid composition analysis gave the expected results.

Biotinylation of the pY Peptide. About 0.7 mg of the PDGFR 1009 1−13 pY peptide [DTSSVL(pY)TAVQPN] was dissolved in 0.6 mL of 100 mM sodium phosphate (pH 8.0) and incubated with a 15-fold molar excess of NHS-LC-biotin [sulfosuccinimidyl-6-(biotinamido)hexanoate] for 2 h at room temperature, at which point Tris was added to a final concentration of 0.1 M. The mixture was incubated for 30 min and then acidified with TFA, and the biotinylated peptide was purified by reverse-phase HPLC. Mass spectral analysis confirmed that there was one mole of biotin per mole peptide.

Preparation of GST Fusion Proteins. GST fusion proteins of SH-PTP2 (GST−SH2, GST−SH-PTP2) and a catalytically inactive form in which Cys^{459} of the catalytic domain has been changed to Ser (GST−SH-PTP2•C→S, GST−PTP2•C→S) have been reported previously (Bastien *et al.*, 1993; Dechert *et al.*, 1994). The SH-PTP2 sequences fused to GST were as follows: full-length fusions, all residues except the first three (i.e., residues 4−593); GST−SH2 (SH2 domains), residues 4−208; and GST−PTP2•C→S (mutated catalytic domain alone), residues 210−593. *Escherichia coli* cells expressing the various GST fusions were grown in Terrific Broth + ampicillin (100 μg/mL) at 37 °C to OD_{595} ~0.6. The temperature of the culture was dropped to 27 °C, and expression was induced with 50 μM IPTG overnight at 27 °C. Cells were harvested and resuspended in ice-cold lysis buffer (PBS containing 1 mM PMSF, 1 mM BAEE, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 5 mM DTT, and 1% Triton X-100). The cells were either frozen in liquid nitrogen and stored at −80 °C or lysed immediately. Lysis was performed using a Bead Beater (Biospec Products) with 0.1 mm diameter glass beads. Fusion protein was purified by adding GT-Sepharose to the cleared lysate and incubating for 1 h at 4 °C with end-over-end mixing. The beads were pelleted and washed extensively with ice-cold PBS. Purity of the preparation was assessed by SDS−PAGE by the method of Laemmli (1970) followed by Coomassie staining. The concentration of fusion protein on the beads was estimated by visual comparison of the Coomassie-stained SDS−PAGE to ovalbumin standards. Thrombin digestion to remove the GST from the GST−SH-PTP2 and GST−SH-PTP2•C→S fusions was carried out on the GT-Sepharose beads essentially as described (Dechert *et al.*, 1994). Fusion proteins were eluted from the GT-Sepharose beads with 10 mM GT.

Real Time Binding Measurements with BIAcore. All experiments were carried out in 20 mM HEPES (pH 7.3), 150 mM NaCl, 5 mM EDTA, 5 mM DTT, and 0.005% Tween 20. All of the proteins were desalted before use in the above buffer. Streptavidin (50 μg/mL) in 20 mM sodium acetate (pH 4.5) was immobilized onto the sensor chip according to the manufacturer's instructions using *N*-hydroxysulfosuccinimide (NHS) and *N*-ethyl-*N*′-(dimethylpropyl)carbodiimide (EDC). The excess reactive groups were blocked using 1 M ethanolamine. A flow rate of 5 μL/min was used for all experiments. Routinely, 15 μL of biotinylated peptide (5 μg/mL) was injected onto the chip. Binding of antiphosphotyrosine monoclonal antibody (injected at 10 μg/mL) resulted in a 1100−1500 change in resonance units in various experiments. The sensor surface was regenerated at the end of each experiment with 100 mM HCl which did not dissociate the high-affinity biotin−streptavidin interaction. This was confirmed by the reproducibility of the change in resonance units upon binding of antiphosphotyrosine monoclonal antibody to the pY peptide on the sensor chip after several cycles of regeneration.

Phosphorylation of Peptide Substrate. A GST-tyrosine kinase fusion protein immobilized on GT-agarose beads[2] was used to phosphorylate the PDGFR 1009 1−13 peptide. Twenty microliters of the beads was washed in reaction buffer (50 mM imidazole pH 7.2, 10 mM DTT, 30 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM sodium orthovanadate, and 0.05% Triton X-100) and mixed with 40 nmol of peptide substrate, 370 μCi of [γ-^{32}P]ATP (3000 Ci/mmol), and 60−80 nmol of unlabeled ATP, in a final volume of 210 μL. The reaction was allowed to proceed overnight at room temperature with end-over-end mixing and then diluted in 50 mM imidazole, pH 7.2, plus 10 mM DTT, with 100% TFA added to a final concentration of 10%. The sample was loaded on a C_{18} column (Extract-Clean), and the column was washed with 0.1% TFA. Peptide was eluted with 0.1% TFA in 50% acetonitrile, dried, and resuspended in dH_2O. The stoichiometry of phosphorylation was about 22%. Quality of phosphopeptide product was assessed by HPLC (data not shown) and phosphoamino acid analysis that identified phosphotyrosine as the only phosphoamino acid species present (data not shown).

Competitive Assay for SH2 Domain Binding. Fusion protein immobilized on GT-Sepharose beads was mixed with GT-agarose or GT-sepharose beads to a concentration of about 22 pmol of fusion protein/μL of beads. Binding assays were carried out using the Millipore MultiScreen Assay

[2] W. R. Huckle and H. S. Earp, manuscript in preparation.

System, which uses 96-well plates with a 0.45 μm Durapore membrane on the bottom of the wells. A typical well contained 12.5 μL of beads with the immobilized fusion protein (22 pmol/μL beads), 0.2 μM ^{32}P-labeled pY peptide, and 75 μg/mL BSA, to a final volume of 100 μL in PBS. Binding was allowed to proceed for 15 min at room temperature with shaking to keep the beads from settling. Unbound peptide was washed away on the Millipore vacuum manifold with two 100 μL washes with ice-cold PBS. The beads and filters were allowed to air dry and then punched out into scintillation vials with the Millipore multiple punch apparatus. Aquasol scintillation fluid was added and samples were counted in a Beckman LS6000IC scintillation counter.

Phosphatase Kinetic Assays. RCM lysozyme was prepared and phosphorylated with [γ-^{32}P]ATP as described (Tonks *et al.*, 1988) using wheat germ lectin-purified human insulin receptor prepared from an overexpressing cell line (Yoshimasa *et al.*, 1990). ^{32}P-Labeled RCM lysozyme was desalted on a PD-10 Sephadex G-25M column from Pharmacia. The tyrosine phosphatase activity was measured by following the release of ^{32}P from ^{32}P-labeled RCM lysozyme. The assay mixture contained 50 mM imidazole, pH 7.2, 5 mM DTT, 1 mM EDTA, 100 mM NaCl, 100 μg/mL BSA, and 1 μM ^{32}P-labeled RCM lysozyme, in a 50 μL volume. Enzyme was added to initiate the reaction (80 ng of full-length or 40 ng of ΔSH2 SH-PTP2), and the mixture was incubated at 25 °C for 10 min. The reaction mixture was stopped by adding 25 μL of cold TCA followed by 25 μL of 10 mg/mL BSA and then incubated on ice for 10 min and centrifuged at 16000*g* for 5 min. Released ^{32}P was determined by adding 75 μL of the supernatant to 10 mL Aquasol scintillant and counting in a Beckman LS6000IC scintillation counter. The reaction rates were linear under these conditions. Typical rates measured were 0.82 nmol/(mg·min) for full-length SH-PTP2 and 19.6 nmol/(mg·min) for ΔSH2 SH-PTP2.

RESULTS

Interaction of PDGFR 1009 pY Peptide with SH-PTP2. The SH2 domains of SH-PTP2 have been shown to interact *in vivo* with the pY residue at position 1009 of the PDGF receptor β chain (Lechleider *et al.*, 1993a). To measure the kinetics of this binding interaction, a 13 amino acid pY peptide centered around phosphotyrosine 1009 was chemically synthesized, biotinylated, and immobilized through streptavidin on the dextran-coated BIAcore sensor chip. The interaction of various proteins with the pY peptide on the sensor chip can be followed continuously by surface plasmon resonance. Figure 1 shows an overlay sensogram when various proteins were injected over the sensor chip. GST−SH2, GST−SH-PTP2•C→S, and anti-phosphotyrosine antibody all bound to the sensor surface whereas GST alone and GST−PTP2•C→S did not show any binding. Dephosphorylation of the phosphopeptide by injecting active tyrosine phosphatase eliminated the binding, and part of the binding could be restored by phosphorylation of the immobilized dephosphorylated peptide with tyrosine kinase (data not shown). These findings confirm that the SH2 domains of SH-PTP2 specifically interact with the pY residue of the PDGFR 1009 pY peptide. It was necessary to use the catalytically inactive Cys^{459} → Ser mutant fusions to prevent the dephosphorylation of the pY peptide by SH-PTP2. In this mutant the active site Cys, which forms a thiol−phosphate intermediate during dephosphorylation, is changed to Ser, rendering it catalytically inactive.

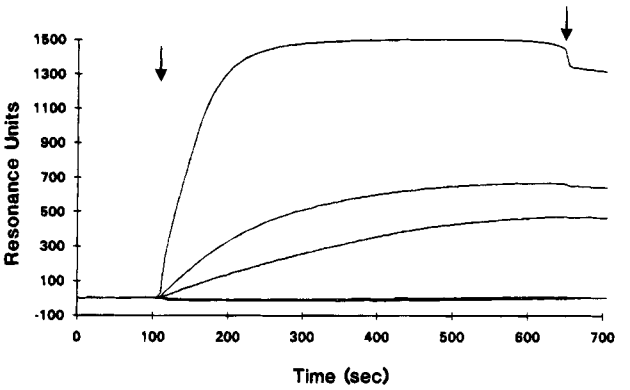

FIGURE 1: Overlay sensograms for the binding of various GST−SH-PTP2 fusions to immobilized PDGFR 1009 1−13 pY peptide. Biotinylated PDGFR 1009 1−13 pY peptide was bound to the sensor surface through immobilized streptavidin as described under Experimental Procedures. All proteins were injected at 10 μg/mL in running buffer. The plots from top to bottom are for anti-phosphotyrosine monoclonal antibody, GST−SH-PTP2•C→S, GST−SH2, GST−PTP2•C→S, and GST. First arrow, injection of protein; second arrow, wash with running buffer.

To determine the association and dissociation rate constants of the SH2 domains, varying concentrations of the SH2 domain-containing protein constructs were injected over the same surface, regenerating the surface at the end of each injection. The overlay plot for the GST−SH2 interaction with the peptide at different concentrations of protein is shown in Figure 2A. In order to determine the association rate constants, the d*R*/d*t* vs resonance units values were plotted (Figure 2B). The slope of the plot of d*R*/d*t* vs concentration of protein injected (Figure 2C) accurately estimates the association rate constant. After the completion of protein injection (second arrow in Figure 2A), when there is running buffer flowing over the sensor surface, the protein appears to remain on the surface; i.e., there is very little apparent dissociation of the protein. Increasing the flow rate did not modify the rate of dissociation. The possibility that the protein actually dissociated from the peptide but bound again to the peptide surface before it was removed by the running buffer was examined by injecting non-biotinylated peptide over the sensor surface (Figure 2D). Increasing concentrations of the non-biotinylated peptide caused a dramatic increase in the rate of dissociation, reaching a maximum at about 100 μg/mL of non-biotinylated peptide. The dissociation followed first-order kinetics. The summary of the association and dissociation rate constants and the K_d for GST−SH2, GST−SH-PTP2•C→S, and SH-PTP2•C→S are shown in Table 1. The rate constants for dissociation for all three proteins were similar, ranging from 0.011 to 0.015 s^{-1}. However, the rate of association for GST−SH2 was 0.6×10^5 $M^{-1}s^{-1}$, approximately 9−12-fold lower than that for GST−SH-PTP2•C→S or SH-PTP2•C→S. The dissociation constant for GST−SH2 was 242 nM while the values for GST−SH-PTP2•C→S and SH-PTP2•C→S were 19 and 18 nM, respectively. The dissociation constants reported here are similar to the values reported earlier using the BIAcore for other SH2 domain-containing proteins (Felder *et al.*, 1993; Panayotou *et al.*, 1993; Payne *et al.*, 1993). The binding was inhibited by preincubation of the proteins with non-biotinylated immobilized peptide or with phosphotyrosine or phenyl phosphate, while non-pY PDGFR

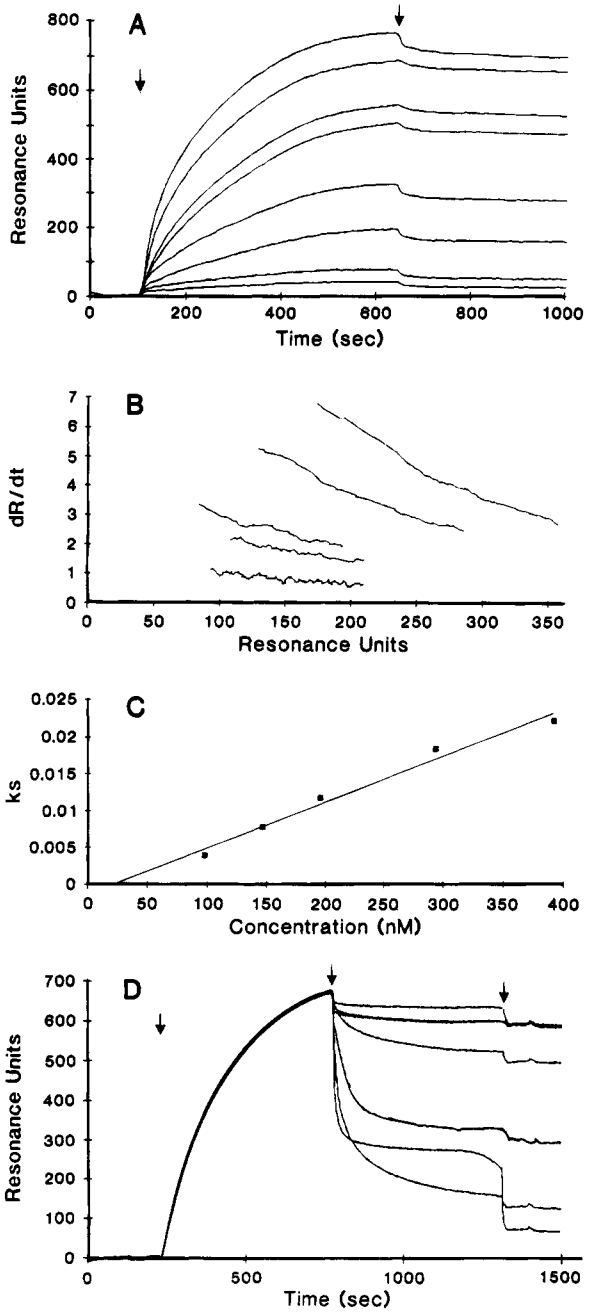

FIGURE 2: Kinetics of binding of GST−SH2 to the PDGFR 1009 1−13 pY peptide. (A) Overlay plots showing change in resonance units with time as varying concentrations of GST−SH2 are injected over the sensor chip. Concentrations of GST−SH2 from top to bottom are (in nM) 392, 294, 196, 147, 98, 49, 25, and 12.5. First arrow, injection of protein; second arrow, wash with running buffer. (B) Plot of the rate of change of resonance units (dR/dt) vs resonance units, as calculated by the BIAcore software. The initial fast change in the refractive index is not included as this is due to the bulk flow of the buffer. (C) Replot of the slopes of the curves in B vs concentration of GST−SH2 injected. (D) Dissociation of bound GST−SH2 from the biotinylated peptide on the sensor chip by injection of varying concentrations of non-biotinylated peptide. GST−SH2 was injected at 196 nM at the point shown by the first arrow. Non-biotinylated PDGFR 1009 1−13 pY peptide was injected in running buffer at the second arrow, using the second loop on the BIAcore, at the following concentrations (from top to bottom, in μg/mL): 0, 0.1, 1, 10, 20, 50, and 100. Third arrow, wash with running buffer.

1009 peptide had no effect on binding (data not shown). The binding of GST−SH2 as measured by the change in resonance units was not affected by mixing GST with GST−SH2 before injection (data not shown); as well, the binding constants for GST−SH-PTP2•C→S and SH-PTP2•C→S are very similar (Table 1). These observations are consistent with the GST fusions binding only as monomers.

Table 1: Association and Dissociation Rate Constants for the Interaction of the SH2 Domains of SH-PTP2 with PDGFR 1009 1−13 pY Peptide

Protein	$k_a \pm$ SE (10^{-5} M^{-1} s^{-1})	$k_d \pm$ SE (s^{-1})	K_d (nM)
GST−SH2	0.6 ± 0.05	0.015 ± 0.001	242
GST−SH-PTP2•C→S	5.7 ± 0.9	0.011 ± 0.0002	19
SH-PTP2•C→S	7.4 ± 0.2	0.014 ± 0.001	18

SH2 Binding Assay. PDGFR 1009 1−13 peptide (DTSS-VLYTAVQPN) was phosphorylated on the tyrosine by tyrosine kinase with [γ-^{32}P]ATP, and the radiolabeled peptide was used as a ligand for binding experiments. Preliminary binding experiments were done by mixing the SH2 domains immobilized on GT-Sepharose beads with the peptide ligand in microfuge tubes. Beads were pelleted by centrifugation, the supernatant was removed, and the beads were washed to remove unbound peptide. However, a large amount of the bound peptide dissociated during the washes; as a result, the signal for peptide retained by the immobilized proteins was only about 5-fold above the background. As an alternative, the Millipore MultiScreen Assay System, using 96-well plates with a 0.45 μm membrane at the bottom of the wells, was adopted. GST fusion protein immobilized on GT-Sepharose beads is incubated with the peptide in the wells, and then unbound peptide is washed away by filtration through the membrane on a vacuum manifold. This method keeps the wash times to a minimum as the washes are immediately removed by vacuum filtration through the membrane. While there is still some dissociation of peptide, the result was a much-improved signal to noise ratio for binding as well as greater reproducibility between replicates. This system also facilitates screening many samples and conditions at the same time. Binding experiments with the various GST fusion proteins immobilized on GT-Sepharose beads show that the binding is specific for the SH2 domain, since binding of the radiolabeled peptide to GST−SH2 and GST−SH-PTP2•C→S is approximately 40- and 150-fold, respectively, greater than to GST or GST-PTP2•C→S (Figure 3A). The binding with the full-length mutant enzyme is 3−4-fold above the binding with the SH2 domains alone. As well, binding to GST−SH2 reaches maximal levels within 5 min after addition of peptide, while for GST−SH-PTP2•C→S, it takes up to 30 min to achieve maximum binding. The time course data also show how reproducible the results are between replicates: with four replicates at each time point, the standard error was generally less than 5% for GST−SH2 and GST−SH-PTP2•C→S.

Unlabeled PDGFR 1009 1−13 pY peptide competed with the ^{32}P-labeled 1−13 peptide for binding to the SH2 domains, as indicated by a decrease in the cpm retained by the immobilized GST−SH2 fusion as the concentration of unlabeled peptide was increased. Using this method, the inhibitory concentration that reduces the binding of the radiolabeled peptide to the SH2 domains by 50% (IC_{50}) can be determined (Figure 3B). The low value (7.5 μM) is consistent with the tight binding of the SH2 domains to this peptide.

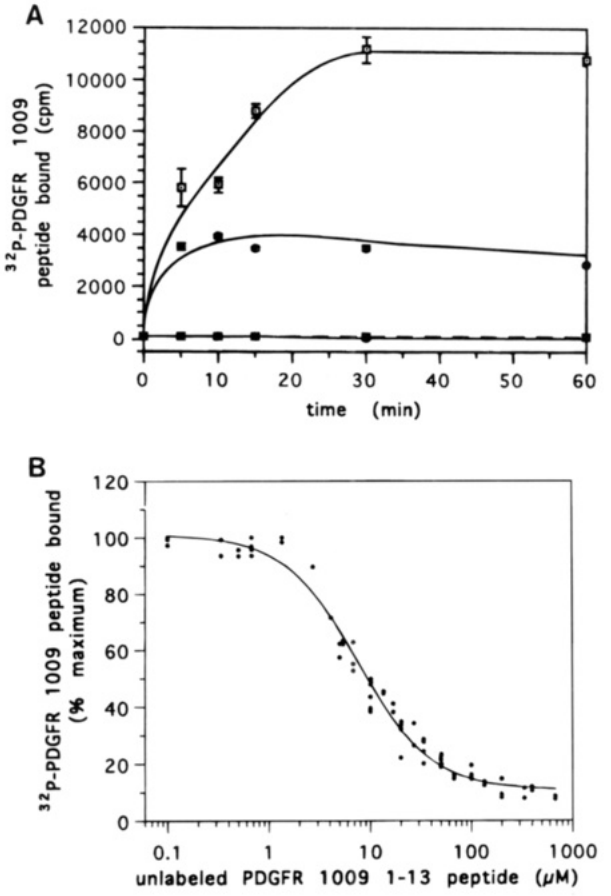

FIGURE 3: SH2 domain binding assay using radiolabeled PDGFR peptide. (A) Time course of binding. ^{32}P-labeled PDGFR 1009 1−13 pY peptide [DTSSVL(pY)TAVQPN] was added to GST (○, GST−SH2 (●), GST−SH-PTP2•C→S (□), or GST−PTP2•C→S (■) immobilized on GT-Sepharose beads as described under Experimental Procedures. "Peptide bound" refers to the cpm retained by the beads after binding and washing, as determined by scintillation counting. The results are the average of four replicates at each time point. (B) Determination of the IC_{50} for the PDGFR 1009 1−13 pY peptide. Binding assays were performed as described under Experimental Procedures. The data plotted are from four separate experiments, with the amount of peptide bound at each concentration expressed as a percent of the maximum binding in that experiment. The IC_{50} was determined by fitting the curve to the equation $y = (m_1 - m_2)/[1 + (x/IC_{50})^{m_4}] + m_2$ where m_1 and m_2 are the minimum and maximum y values, respectively, and m_4 is the slope at the point of inflection (∼−1).

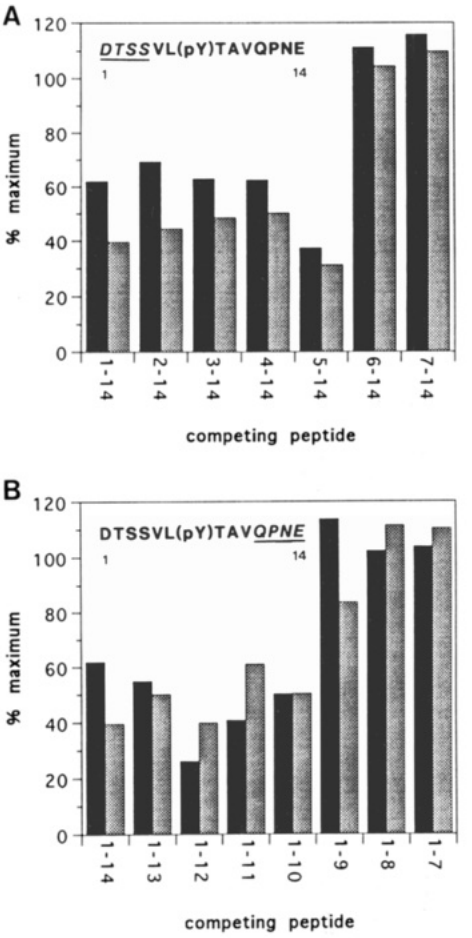

FIGURE 4: Competition for SH2 domain binding by radiolabeled PDGFR peptide with various PDGFR 1009 peptides. Binding competition with various N-terminal (panel A) and C-terminal (panel B) truncated PDGFR 1009 pY peptides. Solid bars, competitive binding assay results; speckled bars, BIAcore assay results. Peptides were added to the competitive assay at a concentration of 8.9 μM, and the assays were performed as described under Experimental Procedures. For the BIAcore assay, GST−SH2 (196 nM) was incubated with the peptides at 17 μM for at least 1 h before injecting over the sensor chip, as described under Experimental Procedures. Results are expressed as percent maximum, which for the competitive assay is the amount of radiolabeled peptide bound as a percent of the control (no competing peptide), and for the BIAcore assay is the maximal increase in resonance units as a percent of the control (no competing peptide).

Determination of Sequence Recognition Elements for SH2 Domains of SH-PTP2. The minimum peptide sequence necessary and sufficient for SH2 domain binding was determined by systematically testing N- and C-terminal truncated versions of the PDGFR 1009 1−14 pY peptide [DTSSVL(pY)TAVQPNE] for their ability to compete with the 1−13 peptide for SH2 domain binding. Both the BIAcore assay and the competitive binding assay were used. In general, the competing peptides were added to the assays at approximately the IC_{50} concentration of the 1−13 peptide. If the truncated peptide is still able to bind to the SH2 domains with a K_d roughly equal to that of the 1−13 peptide, then binding of the 1−13 peptide should be reduced by ∼50% in the presence of the truncated peptide; otherwise, no effect should be observed. In the BIAcore assay, the competing peptides were incubated with GST−SH2 for at least 1 h before injecting over the immobilized PDGFR 1009 1−13 pY peptide on the sensor chip. In the competitive assay, the competing peptides were mixed with the radiolabeled PDGFR 1009 1−13 pY peptide and added to the immobilized fusion proteins together. Both approaches gave very similar results. From the N-terminal truncations (Figure 4A), only the two amino acids immediately N-terminal to the pY residue (Val and Leu at pY−2 and pY−1 respectively) are essential for binding: the 5−14 peptide is the smallest that is still able to compete for binding. Similarly, from the C-terminal truncations (Figure 4B), the three amino acids immediately C-terminal to pY (Thr, Ala, and Val at pY+1, pY+2, and pY+3 respectively) are essential for binding: the 1−10 peptide is the smallest that is still able to compete for binding. Similar results were obtained when GST−SH-PTP2•C→S was used in the two assays (data not shown).

Together, these results suggest that the minimum PDGFR 1009 peptide still able to bind to the SH2 domains would be the 5−10 peptide; i.e., VL(pY)TAV. This peptide is in fact a good ligand (Figure 5A), with an IC_{50} value about

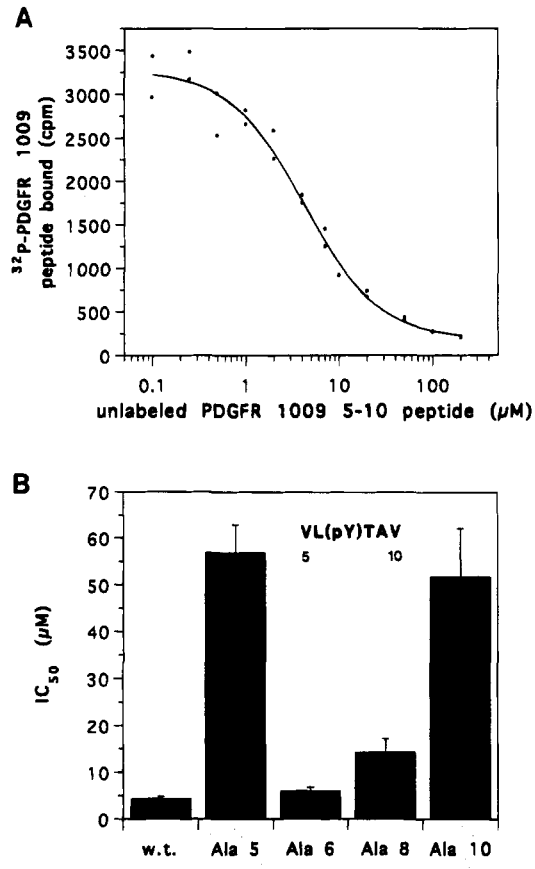

FIGURE 5: Competition for SH2 domain binding by radiolabeled PDGFR peptide with the minimum PDGFR peptide (5−10). (A) Determination of the IC_{50} for the PDGFR 1009 5−10 pY peptide [VL(pY)TAV]. Binding assays and calculations were performed as described under Experimental Procedures and in the legend for Figure 3B. (B) Summary of the IC_{50} values for the 5−10 peptide ("w.t.") and the Ala-substituted derivatives.

2-fold lower than that of the larger 1−13 peptide (see Table 2). In order to identify essential residues or positions for SH2 domain recognition and binding, an Ala scan was performed on this peptide. Four peptides were synthesized, each with one of the residues replaced by Ala (except for pY). The substitutions with the greatest effect on SH2 domain binding and recognition were at positions 5 and 10 (pY−2 and pY+3), as shown by the 12−13-fold increase in the IC_{50} values (Figure 5B and Table 2). The substitution at position 8 (pY+1) had a lesser effect on binding, while the substitution at position 6 (pY−1) had essentially no effect. In addition, double substitution of the Val residues at pY−2 and pY+3 with Ala ("5−10 Ala 5,10") gave rise to a very poor competitor, with an IC_{50} > 500 μM (Table 2).

The second known *in vivo* binding target for SH-PTP2 is IRS-1, at the pY residue 1172 (Kuhné *et al.*, 1993; Sun *et al.*, 1993). An 11-mer peptide centered around phosphotyrosine 1172 was synthesized and tested in the competitive binding assay (Table 2). This peptide was a better competitor than the PDGFR 1009 peptide with an IC_{50} of 0.6 μM. Several other 11-mer peptides corresponding to the known SH2 domain binding sites on IRS-1 for Grb2, p85, and Nck were also tested (Sun *et al.*, 1993; White *et al.*, 1994) (Table 2). Among these, the IRS-1 895 pY peptide (the docking site for Grb2) was an effective competitor. The phosphonomethyl phenylalanine (PMP) derivative of the best binding peptide (IRS-1 1172) was a poor competitor with an IC_{50} about 80-fold higher than that of the pY peptide (Table 2).

Phosphotyrosyl peptide library screening also identified the PDGFR 1021 peptide as a potential binding site for the SH2 domains of SH-PTP2 (Songyang *et al.*, 1993). When this peptide was tested in the competitive binding assay, the IC_{50} was > 500 μM. A 25-mer peptide encompassing the pY residues at positions 1009 and 1021 of PDGFR was only as effective as the PDGFR 1009 1−13 pY peptide, showing that there is no enhancement in binding with the second phosphotyrosine present (Table 2).

Stimulation of SH-PTP2 Activity by SH2 Domain Occupancy. The phosphatase activity of SH-PTP2 has been shown to be stimulated when its SH2 domains are bound to a pY-containing peptide (Sugimoto *et al.*, 1993, 1994; Lechleider *et al.*, 1993b). We decided to test a number of our PDGFR 1009-derived pY peptides for their ability to stimulate the activity of SH-PTP2, to ensure that this correlates with the binding data. ^{32}P-Labeled RCM lysozyme was used as a substrate for the activity assays (Tonks *et al.*, 1988). The amount of stimulation with full-length SH-PTP2 increases rapidly with increasing concentration of exogenous PDGFR 1009 1−13 peptide, reaching a maximum 6−7-fold increase at about 50 μM peptide (Figure 6A, closed circles). SH-PTP2 without the SH2 domains (i.e., the catalytic domain alone) shows no stimulation with exogenous peptide (Figure 6A, open circles). In general, there was a very good correlation between a peptide's capacity to stimulate SH-PTP2 activity and its ability to bind to the SH2 domains (Figure 6B). PDGFR 1009 peptides which bind well to the SH2 domains [as reflected by their IC_{50} values (Table 2)] stimulate the phosphatase activity by 4−5-fold at 50 μM peptide. Peptides that do not bind (7−14 and 1−9) show no stimulation, while peptides that are poor binding ligands (6−14, 5−10 Ala 5, and 5−10 Ala 10) show only about a 2-fold stimulation. The activity of PTP1C (another SH2 domain-containing PTPase) (Plutzky *et al.*, 1992; Matthews *et al.*, 1992; Shen *et al.*, 1991; Yi *et al.*, 1992) was not affected by the above peptides with RCM lysozyme as a substrate (data not shown).

DISCUSSION

SH2 domains provide a means of bringing proteins together in a specific phosphotyrosine-dependent interaction, thereby playing an essential role in the transduction of signals generated both by receptor and nonreceptor PTKs. An understanding of the factors that determine the specificity of this interaction will provide a clearer picture of the nature of the associations and allow predictions of potential interaction partners for a particular SH2 domain. We chose to study the SH2 domains of the protein tyrosine phosphatase SH-PTP2 and to attempt to determine some of the factors that govern its associations with a known *in vivo* binding site, at phosphotyrosine 1009 of the PDGFR β subunit.

SH2 domain binding studies were done using the BIAcore (surface plasmon resonance) and using a competitive binding assay. In the BIAcore assay the peptide was immobilized and free protein was injected and allowed to bind, while in the competitive assay the fusion proteins were immobilized and free peptide was allowed to bind. The fact that two different approaches gave very similar results for the relative affinities of various truncated peptides and PDGFR 1009

Table 2: IC_{50} Values for Various Competitors with the SH2 Domains of SH-PTP2[a]

competitor	sequence	$IC_{50} \pm SE$ (μM)
PDGFR 1009:		
1−13	DTSSVL(pY)TAVQPN	7.5 ± 0.4
1−14	DTSSVL(pY)TAVQPNE	12 ± 2
5−14	VL(pY)TAVQPNE	6.0 ± 1.0
6−14	L(pY)TAVQPNE	97 ± 41
7−14	(pY)TAVQPNE	>500
1−7	DTSSVL(pY)	>500
1−8	DTSSVL(pY)T	>500
1−9	DTSSVL(pY)TA	>500
1−10	DTSSVL(pY)TAV	8.5 ± 1.1
5−10	VL(pY)TAV	4.3 ± 0.5
5−10 Ala 5	AL(pY)TAV	57 ± 6
5−10 Ala 6	VA(pY)TAV	6.0 ± 0.8
5−10 Ala 8	VL(pY)AAV	14 ± 3
5−10 Ala 10	VL(pY)TAA	52 ± 10
5−10 Ala 5,10	AL(pY)TAA	>500
1−13 (nonphospho)	DTSSVLYTAVQPN	>1000
5−10 (nonphospho)	VLYTAV	>1000
PDGFR 1021	GDND(pY)IIPLPD	>500
PDGFR 1009/1021	DTSSVL(pY)TAVQPNEGDND(pY)IIPLPD	9.2 ± 2.6
IRS-1 147	GEDLS(pY)GDVPP	>200
IRS-1 460	EELSN(pY)ICMGG	61 ± 7
IRS-1 608	HTDDG(pY)MPMSP	>200
IRS-1 895	KSPGE(pY)VNIEF	4.6 ± 1.8
IRS-1 939	TGTEE(pY)MKMDL	>200
IRS-1 1172	ENGLN(pY)IDLDL	0.6 ± 0.05
IRS-1 1172 PMP	ENGLN(PMP)IDLDL	48 ± 12
IRP 5	TRDI(pY)ETDYYRK	>200
IRP 9	TRDIYETD(pY)YRK	>200
phenyl phosphate		2500 ± 500
phosphotyrosine	pY	4300 ± 1100

[a] PDGFR 1009-derived peptides are numbered starting with the aspartic acid at position pY−6 as no. 1. In the naming of the PDGFR, IRS-1, and IRP peptides, the number refers to the position of the phosphotyrosine in the native protein. The IC_{50} values were determined as described in the legend to Figure 3B. All phosphopeptides have carboxy amide at the C-terminal except for IRP-5 and IRP-9 which have carboxyl groups.

1−13 pY peptide for SH2 domain binding (Figure 4) provided added validity to the two methods and their results. The SH2 domains of SH-PTP2 have a very high affinity for the PDGFR 1009 pY peptide, as indicated by the submicromolar dissociation constants (Figure 2 and Table 1). Interestingly, the full-length $Cys^{459} \rightarrow$ Ser mutant enzyme has a dissociation constant about 13-fold lower than that for the SH2 domains alone (i.e., with the catalytic domain removed). This is not due to binding of the PDGFR 1009 pY peptide to the inactive catalytic domain, because the catalytically inactive mutant without the SH2 domains (GST−PTP2•C→S) did not bind any peptide (Figure 1). Similar differences in the behavior of the full-length mutant enzyme vs the SH2 domains alone were observed in the competitive assay. The time course for peptide binding to the SH2 domains (Figure 3A) showed that binding to GST−SH2 was maximal within 5 min of the addition of peptide, while for the full-length mutant (GST−SH-PTP2•C→S) it took up to 30 min for maximal binding to be reached. The amount of peptide bound to the full-length mutant at the concentrations used was also 3−4-fold greater than that for GST−SH2. In both types of experiment, the presence of the catalytic domain had an effect on the binding properties of the SH2 domains: the structure of the full-length enzyme was more stable or in a more favourable conformation for binding than the SH2 domains alone.

Structures of SH2 domains complexed with their binding peptides have been determined by X-ray crystallography and NMR (Waksman *et al.*, 1992, 1993; Eck *et al.*, 1993, 1994; Pascal *et al.*, 1994). These studies have shown that most SH2 domains have a conserved Arg residue at the αA2 position in SH2 domains that interacts with the phenyl ring of the pY residue through the ϵ-NH_2 group. However, in SH2 domain-containing PTPases (PTP1C, SH-PTP2, and corkscrew), this Arg is replaced by Gly (Bastien *et al.*, 1993; Freeman *et al.*, 1992; Ahmad *et al.*, 1993; Adachi *et al.*, 1992; Feng *et al.*, 1993; Vogel *et al.*, 1993; Plutzky *et al.*, 1992; Matthews *et al.*, 1992; Shen *et al.*, 1991; Yi *et al.*, 1992; Perkins *et al.*, 1992). This suggests that the interactions with the target peptide for this class of SH2 domain may be different. To determine the important residues, we undertook a systematic approach using N- and C-terminal deletion peptides based on the PDGFR 1009 peptide sequence DTSSVL(pY)TAVQPNE (Figure 4). These studies indicated that the minimum peptide sequence necessary and sufficient for binding to the SH2 domains was VL(pY)TAV. Structural data have shown that with SH2 domains of the *src* family, the residues which make the most important contacts with the SH2 domain are the phosphotyrosine and the residues at positions pY+1 and pY+3 (Waksman *et al.*, 1992, 1993; Eck *et al.*, 1993, 1994). In the case of the PLCγ SH2 domain, contacts have been shown to occur with residues at pY+4 to pY+6 in addition to the contacts shown for the *src* family (Pascal *et al.*, 1994). None of these studies has implicated residues N-terminal to the phosphotyrosine as being important in recognition and binding. In our study, though, it is clear that two residues N-terminal to the phosphotyrosine are necessary for binding to the SH2

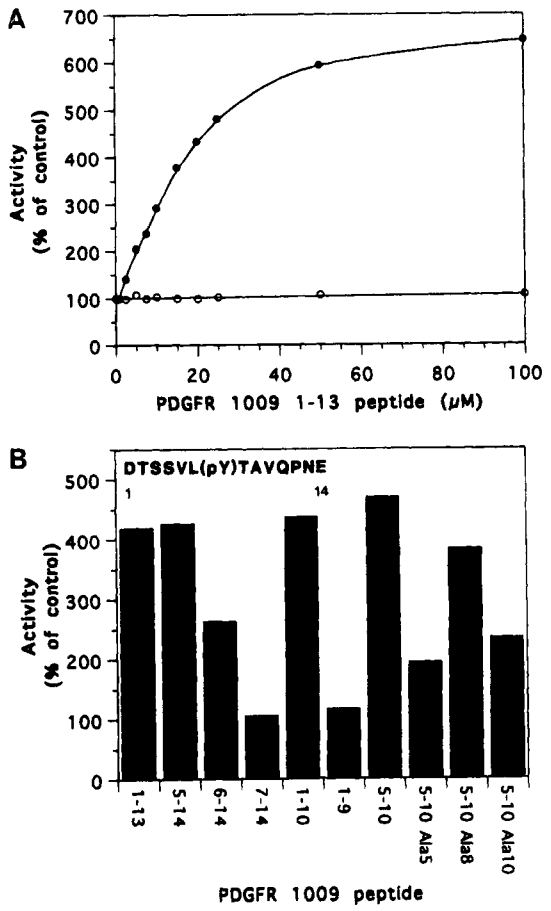

FIGURE 6: Stimulation of SH-PTP2 phosphatase activity by PDGFR 1009-derived peptides. All assays were performed as described under Experimental Procedures. (A) Stimulation of SH-PTP2 phosphatase activity as a function of concentration of exogenous peptide added. PDGFR 1009 1−13 pY peptide was added to the reaction mixture at various concentrations, and the activities were determined from the amount of ^{32}P released. Closed circles, full-length SH-PTP2; open circles, ΔSH2 SH-PTP2 (catalytic domain alone without the SH2 domains). Activities are expressed as percent of control, which is the activity with no exogenous peptide added. (B) Effect of various PDGFR 1009-derived peptides on SH-PTP2 phosphatase activity. The indicated PDGFR 1009 pY peptides were added to the reaction mixture at 50 μM each, and the activities were determined. Activities are expressed as percent of control, which is the activity with no exogenous peptide added.

domains of SH-PTP2 (Figure 4). The Ala scan performed on this minimal peptide (Figure 5) indicated that the Val residue at position pY−2, as well as the Val residue at pY+3, is critical for recognition and binding: replacement of either by Ala results in an approximately 12−13-fold increase in the IC_{50} value for the peptides, and replacement of both Val residues with Ala results in a >100-fold increase (Table 2). The Thr at pY+1 is also important, as replacement by Ala leads to a 3−4-fold increase in the IC_{50} value. Recently, the crystal structure of the amino-terminal SH2 domain of SH-PTP2 complexed with various peptides was determined (Lee *et al.*, 1994), in which there appear to be important interactions between the SH2 domain and the peptide at the pY residue and residues at pY+1, pY+3, and pY+5. Our results with the PDGFR 1009 deletion peptides do not suggest that the pY+5 position is necessary for high-affinity binding (Figure 4 and Table 2). While the crystal structure shows interactions at the pY+5 site, the contribution of this site to the total binding energy may be minor compared to that of the minimum peptide (pY−2 to pY+3). The importance of the contacts between the peptide at pY+5 and the SH2 domain may also depend on the peptide ligand, as the pY+5 residue (Phe) in the IRS-1 895 peptide interacts more extensively with the SH2 domain than does the pY+5 residue (Pro) in PDGFR 1009 (Lee *et al.*, 1994). Interestingly, the crystal structure of the SH2 domain with the PDGFR 1009 peptide also shows that the Val at pY−2 covers the face of the phosphotyrosine ring. This position is occupied by the guanidinium group of the Arg at αA2 in other SH2 domains, but in SH-PTP2 this Arg is replaced by Gly (see above). Thus, it is not unreasonable from the structure that this position in the PDGFR 1009 peptide should also have a role in maintaining a high-affinity interaction with the SH2 domains of SH-PTP2.

Results for the binding with the PDGFR 1021 peptide and other non-PDGFR peptides (Table 2) suggest some basic rules for peptide recognition and binding by the SH2 domains of SH-PTP2. In general, there appear to be three critical positions in addition to the phosphotyrosine: pY+1, pY+3, and pY−2. The Ala-substitutions in the PDGFR 1009 5−10 peptide that changed any of the residues at these positions resulted in a much poorer ligand for SH2 domain recognition and binding. By comparing the sequences of the three peptides that bind well to the SH2 domains (PDGFR 1009, IRS-1 1172, and IRS-1 895), some preliminary predictions can be made. First, a β-branched residue is required at position pY+1: the three peptides have either Thr, Ile, or Val at this position. Second, a hydrophobic residue with an aliphatic side chain is required at position pY+3: the three peptides have either Val, Leu, or Ile at this position. Third, the pY−2 position is critical, with Val, Leu, and Gly present here in the high-affinity binding peptides. A general rule for what residues would be tolerated at this position is not, however, immediately apparent. The other non-PDGFR peptides tested that do not bind have deviations from these rules at the critical positions. Two peptides in particular are noteworthy. First, the IRS-1 147 peptide has a very similar sequence to the IRS-1 1172 peptide in the region from pY−2 to pY+3, including hydrophobic residues at pY−2 and pY+3. But IRS-1 147 has a Gly at pY+1, which is not a β-branched residue: this difference appears to be enough so as not to make IRS-1 147 a binding ligand for the SH2 domains of SH-PTP2. Second, the IRS-1 460 peptide has a β-branched residue (Ile) at pY+1 and a hydrophobic residue (Met) at pY+3 but has a polar residue (Ser) at pY−2. This peptide is poorly recognized by the SH2 domains, with an IC_{50} of 61 μM. These rules are derived from a small number of peptides; binding experiments with more peptides are required to see how broadly applicable these rules are.

A final obvious requirement for recognition and binding is the pY residue. Nonphosphorylated PDGFR 1009 1−13 and 5−10 peptides were not recognized by the SH2 domains of SH-PTP2, with IC_{50} values >1 mM (Table 2). The pY residue alone is not sufficient for binding, though, since both phosphotyrosine and phenyl phosphate are very poor competitors in the binding assay, with IC_{50} values of 4.3 and 2.5 mM, respectively (Table 2). Phosphonomethylphenylalanine (PMP), a phosphotyrosine analogue, does not substitute well for phosphotyrosine in the IRS-1 1172 peptide: the IC_{50} value for this PMP peptide is 48 μM, or 80-fold greater than that for the pY peptide (Table 2). This difference may be because the pK_{A2} of the phosphate of PMP is 7.1 as compared to 5.7 for phosphotyrosine (Domchek *et al.*, 1992); as well,

the substitution of the ester oxygen with a methylene bridge, and the resulting loss of hydrogen bonding between the oxygen and the SH2 domain, may also be important (Waksman *et al.*, 1992; Eck *et al.*, 1993).

With SH2 domain-containing PTPases, a stimulation in phosphatase activity is observed when the SH2 domain is complexed with its peptide binding target (Sugimoto *et al.*, 1993, 1994; Lechleider *et al.*, 1993b). When we tested a number of the PDGFR 1009-derived pY peptides for their ability to stimulate the PTPase activity of SH-PTP2, a good functional correlation was observed with the *in vitro* binding data (Figure 6B). In general, the peptides that were shown to bind to the SH2 domains in the BIAcore and competitive binding assays were also able to stimulate the phosphatase activity of SH-PTP2 by as much as 5−7-fold at the concentrations used in the experiments. This stimulation occurred through the SH2 domains, since the catalytic domain alone without the SH2 domains was not stimulated by the addition of exogenous peptides. In fact, removing the SH2 domains in itself results in a higher specific activity (Dechert *et al.*, 1994), suggesting that the SH2 domains, when not bound to a peptide target, inhibit the phosphatase activity. Therefore, the stimulation observed with the full-length enzyme is really a relief of inhibition. Another SH2 domain-containing PTPase, PTP1C, was not stimulated by the addition of peptides that are bound by the SH2 domains of SH-PTP2, showing that the stimulation by these peptides is specific for SH-PTP2. This is not surprising since different SH2 domains generally recognize different peptide sequences: peptides that are bound by the SH2 domains of SH-PTP2 would not necessarily be bound by the SH2 domains of PTP1C. It is interesting to note that binding of peptides at the SH2 domains stimulates phosphatase activity of SH-PTP2, and that the presence of the catalytic domain enhances binding of target peptide to the SH2 domains (see above). Perhaps both the SH2 domain and the catalytic domain are able to allosterically modulate each other's function.

While this work was being completed, another study on the binding of the SH2 domains of SH-PTP2 was presented (Case *et al.*, 1994) in which a binding assay with ^{125}I-labeled PDGFR 1009 peptide was used, with competitor peptides derived from phosphotyrosine sites in PDGFR, EGFR, and IRS-1. The nature of this assay resulted in a signal to noise ratio of only about 5 or 10 to 1, while the assay described in this paper has a signal at least 40-fold above the background, resulting in a much higher sensitivity. The use here of a ^{32}P-labeled peptide also has the advantage of not needing to modify the peptide substrate for the assay, as the radioactive phosphate is incorporated exactly where the nonradioactive phosphate would be. ^{125}I labeling requires an ϵ-amino group for the best labeling, and if the peptide does not have a Lys residue in the native sequence, this must be added to the peptide substrate. The authors of the other study concluded that the sequence recognition requirements for the N-terminal SH2 domain were, in addition to the phosphotyrosine, (i) a β-branched residue at pY+1 and (ii) a hydrophobic residue at pY+3. Our results using both SH2 domains together support this finding, but in addition to this, the pY−2 position is shown to be critical for recognition and binding. This is the first report of a specific requirement in the region N-terminal to the phosphotyrosine: all other reports have only identified residues C-terminal to the phosphotyrosine. From the truncated PDGFR 1009 peptide studies, it was determined that the minimum peptide necessary and sufficient for SH2 domain recognition and binding is the region from pY−2 to pY+3. This is clearly different from the SH2 domains of *src*-family kinases and of PLCγ and may suggest yet another class of binding mode. Similar studies with other SH2 domains, from both known proteins and ones yet to be identified, will help to clarify the rules that govern the recognition of SH2 domain binding targets and thus explain how specificity is maintained by a small structural element that is used in many different situations and pathways in a cell. Deciphering how this is accomplished may provide clues toward an understanding of the signaling mediated by this class of multifunctional PTPases.

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