Alternative Mode of Binding to Phosphotyrosyl Peptides by Src Homology-2 Domains†

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ABSTRACT: Src homology-2 (SH2) domains recognize specific phosphotyrosyl (pY) proteins and promote protein−protein interactions. In their classical binding mode, the SH2 domain makes specific contacts with the pY residue and the three residues immediately C-terminal to the pY, although for a few SH2 domains, residues N-terminal to pY have recently been shown to also contribute to the overall binding affinity and specificity. In this work, the ability of an SH2 domain to bind to the N-terminal side of pY has been systematically examined. A pY peptide library containing completely randomized residues at positions −5 to −1 (relative to pY, which is position 0) was synthesized on TentaGel resin and screened against the four SH2 domains of phosphatases SHP-1 and SHP-2. Positive beads that carry high-affinity ligands of the SH2 domains were identified using an enzyme-linked assay, and the peptides were sequenced by partial Edman degradation and matrix-assisted laser desorption ionization mass spectrometry. The N-terminal SH2 domain of SHP-2 binds specifically to peptides of the consensus sequence (H/F)XVX-(T/S/A)pY. Further binding studies with individually synthesized pY peptides show that pY and the five residues N-terminal to pY, but not any of the C-terminal residues, are important for binding. The other three SH2 domains also bound to the library beads, albeit more weakly, and the selected peptides did not show any clear consensus. These results demonstrate that at least some SH2 domains can bind to pY peptides in an alternative mode by recognizing only the residues N-terminal to pY.

Src homology-2 (SH2)[1] domain is a small protein module consisting of ∼100 amino acids. It was first identified in the N-terminal regions of Src family protein tyrosine kinases and subsequently found in >100 human proteins, many of which are involved in intracellular signaling processes (*1*, *2*). SH2 domains recognize specific phosphotyrosyl (pY) residues in their partner proteins, promoting protein−protein interactions. The sequence specificity of an SH2 domain−pY protein interaction is primarily determined by the pY residue and the three residues immediately C-terminal to pY (*3*). The structural basis for the specific interaction has been very well-studied (*4*−*6*). A key interaction, which is common to all SH2 domains, is the insertion of the pY side chain into a deep pocket in the SH2 domain, where an invariant arginine residue (Arg βB5) forms a bidentate interaction with the pY phosphate group. Additional binding energy is provided by interactions between amino acids adjacent to pY, particularly the three residues immediately C-terminal to pY, and the less conserved surface of the SH2 domain. This latter interaction also governs the selectivity of a given SH2 domain in binding to a specific pY partner.

More recent studies have revealed that for some SH2 domains, the amino acid residues N-terminal to pY can also significantly affect the affinity and specificity of the interactions. For example, high-affinity binding to the SH2 domains of protein tyrosine phosphatases SHP-1 and SHP-2 requires aliphatic, hydrophobic residues (e.g., leucine, isoleucine, valine, and threonine) at the −2 position (2 residues N-terminal to pY, which is defined as position 0) of a pY peptide (*7*, *8*). The SH2 domains of SAP/SH2DIA and EAT-2 make specific interactions with residues on both the N- and C-terminal sides of pY (positions −3 to +5) (*9*). These observations raise the question whether interaction with residues N-terminal to the pY residue is a common feature of all SH2 domains and whether an SH2 domain can associate with a pY peptide with high affinity by recognizing only the pY and its N-terminal residues in a manner analogous to the phosphotyrosine-binding (PTB) domains (*10*). To the best of our knowledge, no systematic studies have been carried out to specifically address these issues.

In this work, we have developed a combinatorial library method to systematically assess the ability of SH2 domains to interact with the N-terminal sequences of pY peptides. A pY peptide library that contains five randomized residues N-terminal to pY and an invariant C-terminal sequence was synthesized and screened against the four SH2 domains of protein tyrosine phosphatases SHP-1 and SHP-2. The results show that certain SH2 domains can indeed bind to pY

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[1] Abbreviations: SH2, Src homology 2; pY, phosphotyrosyl; MBP, maltose-binding protein; Nle, norleucine; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; MALDI MS, matrix-assisted laser desorption ionization mass spectrometry; GST, glutathione-S-transferase; SA-AP, streptavidin-alkaline phosphatase; PITC, phenylisothiocyanate; Nic-OSU, *N*-hydroxysuccinimidyl nicotinate.

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peptides in an alternative mode, by recognizing residues N-terminal to the pY.

EXPERIMENTAL PROCEDURES

Materials. *N*-Hydroxysuccinimidyl nicotinate (Nic-OSU), peptide synthesis reagents, and resins were purchased from Peptides International, Inc. (Louisville, KY), Advanced ChemTech (Louisville, KY), or Nova Biochem (Switzerland). 5-Bromo-4-chloro-indolyl-phosphate (BCIP), α-cyano-4-hydroxycinnamic acid, phenylisothiocyanate (PITC), and *N*-hydroxysuccinimidobiotin were purchased from Sigma Chemical Co. (St. Louis, MO), and streptavidin alkaline phosphatase (SA-AP) was purchased from Prozyme (San Leandro, CA). Gelatin was purchased from Bio-Rad, and NHS-dPEG$_4$-biotin was from Quanta BioDesign, Ltd. (Columbus, OH). Other chemicals were purchased from Aldrich Co. (St. Louis, MO).

Expression and Purification of SH2 Domains. N-terminally six-histidine-tagged SH2 domains and their fusion proteins with maltose-binding protein (MBP−SH2) were expressed and purified as previously described (*11*−*13*). Biotinylation of the SH2 domains was carried out by treating the MBP−SH2 protein (180−800 μM) in 50 mM sodium phosphate, pH 7.0, and 150 mM NaCl with 2 equiv of N-hydroxysuccinimidobiotin for 30 min at room temperature. The sample was passed through a Pharmacia G-25 Fast-Desalting column equilibrated in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM β-mercaptoethanol, and 1 mM EDTA to remove any free biotin. The resulting protein was concentrated in a Centriprep-10 concentrator and quickly frozen in the presence of 33% glycerol. Protein concentration was determined by the method of Bradford (*14*) using bovine serum albumin as standard.

Library Synthesis. The pY peptide library, AXXXXX-pYAABBRM-Resin (where X is Nle or any of the 18 natural amino acids except for Met and Cys and B is β-alanine), was synthesized on 2 g of TentaGel S NH_2 resin (90 μM, 0.3 mmol/g) using standard Fmoc/HBTU/HOBt chemistry. The random positions were generated by the split-pool synthesis method (*15*−*17*), and the coupling reactions were carried out with 5 equiv of reagents for 2 h and repeated once to ensure complete reaction. To facilitate peptide ladder sequencing by mass spectrometry, a small amount of chain-termination products were generated by the addition of a small amount of capping reagents to the coupling reactions (along with the individual amino acids) during the synthesis of the randomized region (*18*). The capping reagents used were *5% N*-acetylalanine for Nle and 5% *N*-acetylglycine for Leu and Lys.

Library Screening. The pY library (50 mg) was exhaustively washed with CH_2Cl_2, MeOH, H_2O, and a blocking buffer (30 mM Hepes, pH 7.0, 200 mM NaCl, and 0.01% Tween 20) containing 0.1% (w/v) gelatin. After incubation for 1 h in the above buffer, the beads were suspended in 0.8 mL of a binding buffer (30 mM Hepes, pH 7.4, 150 mM NaCl, and 0.005% Tween 20) containing 10−50 nM biotinylated MBP−SH2 domain and incubated for 6 h at 25 °C with constant mixing. The beads were quickly washed with 1 mL of the binding buffer and resuspended in 0.8 mL of the binding buffer containing ∼12 nM SA-AP and 25 mM potassium phosphate. The mixture was shaken at 4 °C for 10−15 min and quickly washed with 3 × 2 mL of the binding buffer and 2 × 2 mL of SA-AP reaction buffer (30 mM Tris-HCl, pH 8.5, 100 mM NaCl, 5 mM $MgCl_2$, and 0.02 mM $ZnCl_2$). The beads were suspended in 1.2 mL of SA-AP reaction buffer containing 1.4 mM BCIP and incubated at room temperature. Typically, the staining reaction was allowed to proceed for 30−60 min before being quenched by the addition of 1 mL of 8 M guanidine hydrochloride. The beads were washed exhaustively with water, and positive beads were readily identified by their intense turquoise color and manually removed from the library with a micropipet with the aid of a low-power microscope. A control screening was performed with biotinylated MBP (no SH2) under the same conditions but resulted in no colored beads.

Peptide Sequencing. The positive beads from above were pooled, transferred into a 5-mL fritted glass column, and washed exhaustively with methanol, water, and a mixture solvent (pyridine/water/triethylamine, 2:1:0.001). The beads were then subjected to 6 cycles of partial Edman degradation plus a final treatment with Nic-OSU to generate a peptide ladder on each bead, as previously described (*18*). The PITC/Nic-OSU ratio employed ranged from 9:1 to 6:1. After exhaustive washing with methanol, CH_2Cl_2, and TFA, the beads were treated with 1 mL of TFA containing methyl sulfide (20 μL) and ammonium iodide (20 mg) on ice for 15 min to reduce any oxidized methionine. The beads were then transferred into individual microcentrifuge tubes, and each bead was treated with 20 μL of a 70% TFA solution containing 40 mg/mL CNBr for 20 h in the dark. The solvent and excess CNBr were removed under vacuum, and the released peptides from each bead were dissolved in 5 μL of 0.1% TFA in water. One microliter of the peptide solution was mixed with 3 μL of a saturated α-cyano-4-hydroxycinnamic acid solution in a mixture solvent (CH_3CN/H_2O/TFA, 50:50:0.1), and 1 μL was loaded onto a 384-well sample plate. The samples were analyzed on a Bruker Reflex III matrix-assisted laser desorption ionization mass spectrometer (MALDI MS). Peptide sequences were manually determined from the MS spectra.

Synthesis of Phosphotyrosyl Peptides. Phosphotyrosyl peptides were synthesized on Rink resin (0.015−0.2 mmol scale) using standard Fmoc chemistry. Crude peptides (∼80% purity as judged by analytical HPLC) were used directly in stimulation assays of SHP-2. For BIAcore analysis, the crude peptides (5 mg) were dissolved in 0.5 mL DMF containing 3% *N*-methylmorpholine and treated with NHS-dPEG$_4$-biotin (10 mg) for 3 h at room temperature. The biotinylated peptides were purified by reversed-phase HPLC on a semipreparative C_{18} column prior to use. The identity of all peptides was confirmed by MALDI mass spectrometric analysis.

Stimulation of SHP-2 Activity by pY Peptides. A typical assay reaction (total volume of 50 μL) contained SHP-2 (70 nM), 10 mM *p*-nitrophenyl phosphate (*p*NPP), 100 mM Hepes, pH 7.4, 1.0 mM β-mercaptoethanol, 1.0 mM EDTA, 150 mM NaCl, and 0−2000 μM pY peptide. The reaction was allowed to proceed for 30 min at room temperature before being quenched with 950 μL of 1 M NaOH, and the absorbance at 410 nm was measured on a UV−vis spectrophotometer. Stock solutions of pY peptides were made in DMSO at a concentration of 40−100 mM. Peptide concen-

trations were determined by hydrolysis of pY to completion with alkaline phosphatase followed by the malachite green assay of the released inorganic phosphate (*19*). All reactions were carried out in triplicates.

Surface Plasmon Resonance (SPR). All measurements were made on a Pharmacia BIAcore 3000 instrument. Biotinylated pY peptides were immobilized onto streptavidin-coated sensorchips by passing a 0.1 μM pY peptide solution in HBS buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20) over the surface of a chip. The amount of immobilized pY peptide was determined by saturating the chip surface with six-histidine-tagged SH2 domain and measuring the maximal response units. Data for secondary plot analysis were generated by passing increasing concentrations (0−100 μM) of an SH2 domain in HBS buffer over the chip surface for 2 min at a flow rate of 15 μL/min. A sham flow cell with no attached peptide was used to account for any signal generated due to the solvent bulk or any other effect not specific to the SH2−peptide interaction. Between runs, regeneration of the chip surface was facilitated by using the running buffer supplemented with 0.025% SDS and 0.019% NP-40. Data analysis was carried out by plotting the response at equilibrium, obtained by subtracting the response of the sham flow cell from that of the pY peptide flow cell, against the SH2 protein concentration. The data were fit to the following equation:

$$RU_{eq} = RU_{max}[SH2]/(K_D + [SH2])$$

where RU_{eq} is the measured response and RU_{max} is the maximum response.

RESULTS

Library Design, Synthesis, and Screening. To test whether SH2 domains can recognize N-terminal sequences of pY peptides, a peptide library, AXXXXXpYAABBRM-resin (X = norleucine or any of the 18 natural amino acids except for Met and Cys; B = β-alanine), was constructed. The five residues immediately N-terminal to the pY are randomized, whereas two invariant residues (AA) are placed on the C-terminal side. The C-terminal methionine permits the peptides to be released from the resin by CNBr treatment prior to sequencing, whereas the C-terminal arginine provides a fixed positive charge that will improve the solubility of the peptides and increase the sensitivity for sequencing by mass spectrometry. The two β-alanines add some flexibility to the peptides, making them more accessible to a protein target. The N-terminal alanine was added to minimize any bias caused by interactions between an SH2 domain and the positively charged N-terminus. Methionine is excluded from the randomized region to avoid internal cleavage during CNBr treatment and is replaced by the isosteric norleucine residue. The library was synthesized on TentaGel S NH_2 resin (2.86×10^6 beads/g) using the split-pool method (*15*−*17*), with each bead carrying a unique sequence (~100 pmol of peptide on each bead). This method ensures equal representation of all possible sequences in the library.

The library (theoretical diversity = 19^5 or 2.5×10^6) was screened for binding to the SH2 domains of SHP-1 and SHP-2. These SH2 domains, constructed as maltose binding protein fusion, were biotinylated on a surface lysine residue(s) by treatment with *N*-hydroxysuccinimidobiotin. Binding of the biotinylated SH2 domain to a bead that carries a high-affinity pY peptide for the SH2 domain recruits SA-AP conjugate to the surface of that bead. Upon the addition of BCIP, the bound alkaline phosphatase cleaves BCIP into an indole, which instantaneously dimerizes in air into indigo, resulting in a turquoise precipitate deposited on the bead surface. As a result of this reaction cascade, a bead carrying a specific SH2 ligand would become colored. The colored beads were manually removed from the library, and the bound peptides were individually sequenced by partial Edman degradation and MS analysis (*18*).

Table 1: Peptide Sequences Selected against SHP-1 and SHP-2 SH2 Domains

SHP-2 N-SH2		SHP-2 C-SH2		SHP-1 N-SH2		SHP-1 C-SH2
HVAYA	FSPRM	AYHAY	FPFYR	DLMKV	HHSQG	IKTYY
HFVNA	VHVPS	WNRGH	LIHYR	HKVYV	HPQFG	WYKMY
HYVIA	VLVYS	RYHGY[a]	FVHYR	HISNV	HWSMS	HNAMF
HHVIS	VTVYT	HKHGY	YFIHR	ALHTV	YRFNS	ASFKF
HHVIS	VFVQA	HRFGY	YMHHR	NVAIM	MLLHS	HTHGF
HFVRS	LQVVS	HAWGF	HWNMR	HIYVM	MWMVT	FYAQF
HAVVS[a]	LIVVT	FTAGF	HNYSR	HSFMM	IIAFT	HMAWF
HYYTS	LILRT	QKSGF	HLGKR	HITAM	YIIRT	TIMVH
HMVMT[a]	LFIHT	YYSWF	HYDWR	HTNGM	MFFHD	YAQVH
HIVMT	IQVVT	IRHPY	HYSVR	LHWGM	MNRQH	TWFWH
HIVPT	IFVRQ	HWLVY	HGTIR	MAAGM	SRWSH	VFRHP
HSVPT	MFVRY	THRHY	RYHIG	MRTMM	TMMVH	YAQGM
HRVYT		RFTWY	NTFIA	MFKNM	ISVNH	TQYYG
HFAAT		HRWYY	YAHYA	MTHQM	MHHNQ	YQYYG
HVLVT		MAVYH	WRYYA	MMRQM[a]	RMDGQ	AWPHS
HHFMF		RYMVH	YYHYM	IIVQM	HVMLF	NYHYS
HYNWY		RYTNH	IHRYM	LNSMM	IHFPY	YYAFS
FHPQY		RFMYQ	HGFFM	YGFSM	RQRKY	NRWPA
FSVQY		AQQYQ	HHLWM	RNKTM	HDDMY	YYHWA
FYARS		FVRWQ	HRHWM	LLRTM	HRHDY	FDRWT
FYVRT		AGRKQ	HRFLM	FRQRM	HRASK	AYQFT
FVIRT		WHFFN	NRYHM	ARRFM	HRFGR	SYFHV
FRVFT		FWWYS	RYHWV	HHLWM	YMVAR	YPMKV
FIVIT		VTNHT	YYNKV	IFQMA	MIRMR	WAHPQ
FVPFT		NHRHT	HRWII	FTTAA	YWMHR	RTYLQ
FMMQT		FWDIT	NWTKI	YRNRA	AMFHR	
FTVYG				RRFVP	AAAHR	
FYVPA				HRLMG	MMMFR	

[a] Peptides selected for individual synthesis and binding studies.

Analysis of Selected Sequences. Approximately 100 mg of resin (~2.86×10^5 beads) was screened against each SH2 domain (final concentration of 10−50 nM), typically resulting in 30−60 colored beads. We have previously demonstrated that the sequence specificity of a modular domain can be unambiguously determined by screening just a small fraction of the library (e.g., 10%) (*13*, *20*). For the N-terminal SH2 domain of SHP-2, a total of 40 sequences were obtained from the most colored beads (Table 1). It strongly prefers a valine at the −3 position; more than 60% of the selected sequences contain a valine at this position (Figure 1). The next most preferred amino acids are proline (10%) and alanine (8%) which, like valine, also contain small hydrophobic side chains. At the −5 position, histidine and phenylalanine are most preferred, followed by hydrophobic aliphatic amino acids (e.g., valine, leucine, and isoleucine). The N-SH2 domain prefers a small residue such as threonine, serine, and alanine at the −1 position. At the −4 and −2 positions, many amino acids are well tolerated (Figure 1). Thus, this SH2 domain binds specifically to pY peptides of the consensus (H/F)XVX(T/S/A)pY, where X represents a variety of amino acids (though not necessarily any amino acid) (Figure 1). The screening results for the C-SH2 domain of SHP-2 and the N- and C-SH2 domains of SHP-1 were

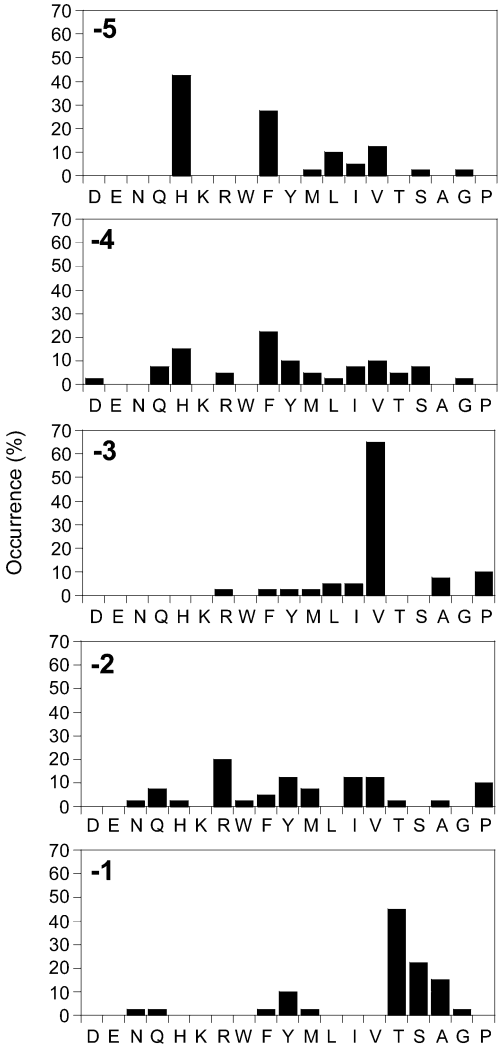

FIGURE 1: Sequence specificity of the SHP-2 N-SH2 domain for binding to pY peptides in the alternative mode. Each panel shows the appearance frequency of 19 amino acids at a given position N-terminal to the pY residue (total 40 sequences). M, norleucine.

more complex (Table 1). Although all three SH2 domains resulted in colored beads, color development required longer staining time or higher SH2 domain concentration, indicating that they bind to the library peptides with lower affinities. For each of these SH2 domains, multiple, highly similar sequences were selected from the library, however, the selected sequences were still too diverse to produce a clear consensus sequence(s). It appears that these SH2 domains are capable of interacting with multiple classes of peptides of different consensus sequences.

Activation of SHP-2 Activity by pY Peptides. To test whether the selected pY peptides actually bind to SHP-2 SH2 domains, we examined their ability to stimulate the catalytic activity of SHP-2. It has previously been established that the N-terminal SH2 domain of SHP-1/-2 directly binds to and inhibits their phosphatase domain (*11*, *21*). Binding of a pY peptide to the N-terminal SH2 domain disengages the intramolecular SH2−PTP complex and stimulates the enzymatic activity. There is a general correlation between the binding affinity of a pY peptide to the N-terminal SH2 domain and its ability to stimulate the enzymatic activity, thus, providing a simple method to screen pY peptides for binding to the N-terminal SH2 domain. Two representative

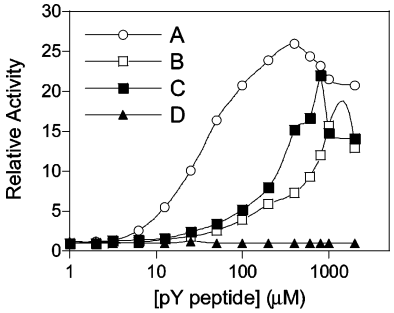

FIGURE 2: Concentration-dependent stimulation of SHP-2 activity by selected pY peptides. The peptides used were (A) Ac-LVpYATILNBBKR-NH_2, (B) Ac-HMVMTpYAABK-NH_2, (C) Ac-HMVMTpY-NH_2, and (D) Ac-HMVMTYAABK-NH_2.

peptides from Table 1, Ac-HMVMTpYAABK-NH_2 and Ac-HAVVSpYAABK-NH_2, which were both selected against SHP-2 N-SH2 domain, were chemically synthesized. To confirm that the SH2 domain recognizes only the pY and its N-terminal sequence, their truncation variants that contain no amino acid C-terminal to pY, Ac-HMVMTpY-NH_2 and Ac-HAVVSpY-NH_2, were also prepared. All four pY peptides activated SHP-2 in a concentration-dependent manner, whereas the corresponding unphosphorylated peptide (Ac-HMVMTYAABK-NH_2) had no effect (Figure 2 and data not shown). Both peptide Ac-HAVVSpYAABK-NH_2 and its truncation variant (Ac-HAVVSpY-NH_2) resulted in a maximal activation of ~20-fold, and the half-maximal SHP-2 activity was reached at 500 μM pY peptide (EC_{50}). For peptide Ac-HMVMTpYAABK-NH_2, its C-terminal truncation variant was actually more potent than the full-length peptide (EC_{50} = 300 vs 600 μM) (Figure 2). Peptide Ac-LVpYATILNBBKR-NH_2, which binds to both N- and C-SH2 domains of SHP-2 in the classical manner (*13*), was used as a positive control. This peptide stimulated SHP-2 activity by 26-fold, with a lower EC_{50} value (~60 μM). The higher potency of the latter peptide is likely due to its ability to simultaneous binding to both N- and C-SH2 domains, which causes more effective activation (*13*, *21*). These results suggest that the selected pY peptides are indeed capable of binding to the N-SH2 domain. Further, the interaction only requires the pY residue and the residues N-terminal to pY.

Affinity Measurement of pY Peptides by SPR. One of the SHP-2 N-SH2 domain-binding peptides, Ac-HMVMT-pYAABK-NH_2, was selected for further analysis by SPR technique. This peptide and its unphosphorylated control peptide were labeled with a biotin at their C-terminal lysine side chain through a flexible, water-soluble poly(ethyleneglycol) linker and loaded onto a streptavidin-coated sensorchip. Its C-terminal truncation variant was similarly labeled with a biotin at its N-terminus [biotin-PEG_4-K(α-Ac)-BHMVMTpY-NH_2] and immobilized onto the chip (in a separate flow channel). Solutions containing N-terminally six-histidine-tagged SH2 proteins were passed over the chip surface, and binding to the immobilized peptides was monitored by the increase in equilibrium response units (RU_{eq}). Both the full-length pY peptide and its C-terminal truncation variant bound to SHP-2 N-SH2 domain, but not the unphosphorylated control peptide (Figure 3). To determine whether the selected pY peptides bind to the N-SH2 domain by occupying the canonical pY-binding pocket, competition experiments were performed using the conven-

Table 2: Dissociation Constants (K_D, μM) of Selected pY Peptides toward Various SH2 Domains

peptide	SHP-2		SHP-1		Src
	N-SH2	C-SH2	N-SH2	C-SH2	
Ac-HMVMTpYAABK(-NH_2)-PEG_4-biotin[a]	3.4 ± 0.2	93 ± 6	17 ± 4	770 ± 13	8.2 ± 1.7
Ac-MMRQMpYAABK(-NH_2)-PEG_4-biotin[b]	130 ± 8	180 ± 12	33 ± 6	740 ± 98	59 ± 11
Ac-RYHGYpYAABK(-NH_2)-PEG_4-biotin[c]	200 ± 19	120 ± 7	56 ± 8	510 ± 57	6.5 ± 0.9
biotin-PEG_4-K(Ac)BH(Nle)V(Nle)TpY-NH_2	3.6 ± 0.3	ND[d]	ND[d]	ND[d]	ND[d]
biotin-PEG_4-K(Ac)BHMVMTpY-NH_2	3.2 ± 0.2	ND[d]	ND[d]	ND[d]	ND[d]
biotin-PEG_4-K(Ac)BHMVMApY-NH_2	7.8 ± 0.4	ND[d]	ND[d]	ND[d]	ND[d]
biotin-PEG_4-K(Ac)BHMVATpY-NH_2	6.9 ± 0.3	ND[d]	ND[d]	ND[d]	ND[d]
biotin-PEG_4-K(Ac)BHMAMTpY-NH_2	6.9 ± 0.5	ND[d]	ND[d]	ND[d]	ND[d]
biotin-PEG_4-K(Ac)BHAVMTpY-NH_2	5.9 ± 0.8	ND[d]	ND[d]	ND[d]	ND[d]
biotin-PEG_4-K(Ac)BAMVMTpY-NH_2	17 ± 1	ND[d]	ND[d]	ND[d]	ND[d]
Ac-LVpYATILNBKR(-NH_2)-PEG_4-biotin[a]	1.9 ± 0.1	2.0 ± 0.2	1.6 ± 0.5	5.2 ± 0.5	3.5 ± 0.2
biotin-PEG_4-K(Ac)BHMVMTpYATIL-NH_2	1.0 ± 0.1	ND[d]	ND[d]	ND[d]	ND[d]

[a] Peptides selected against the SHP-2 N-SH2 domain. [b] Peptide selected against the SHP-1 N-SH2 domain. [c] Peptide selected against the SHP-2 C-SH2 domain. [d] ND, not determined.

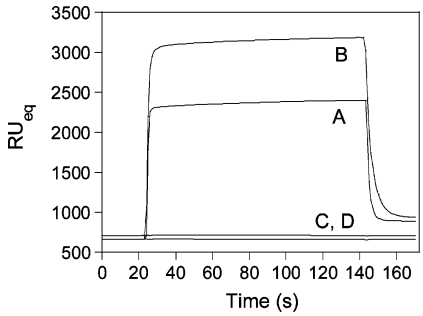

FIGURE 3: SPR analysis of the interactions between the SHP-2 N-SH2 domain and various immobilized peptides. (A) Sensogram resulted from the injection of N-SH2 domain (7.8 μM) over the immobilized peptide Ac-HMVMTpYAABK-dPEG$_4$-biotin. (B) Injection of 7.8 μM N-SH2 domain over immobilized biotin-dPEG$_4$-K(Ac)BHMVMTpY-NH_2. (C) Injection of 7.8 μM N-SH2 domain over immobilized Ac-HMVMTYAABK-dPEG$_4$-biotin. (D) N-SH2 domain (7.8 μM) was preincubated with peptide LVpYATIL (50 μM) and then passed over the immobilized peptide Ac-HMVMTpYAABK-dPEG$_4$-biotin. All sensograms shown are after subtraction of background signal, generated by flowing the same protein solution over a blank channel (no immobilized peptide).

tional SH2 domain-binding peptide Ac-LVpYATILNBBKR-NH_2 as the competitor. Thus, the SHP-2 N-SH2 domain (7.8 μM) was preincubated with peptide pYATI (50 μM) and then tested for binding to the immobilized pY peptides. No binding was observed (Figure 3), suggesting that peptides HMVMTpY and pYATI share the same pY-binding site on the SH2 domain.

The dissociation constants (K_D) for the interactions were measured by passing increasing concentrations of SH2 protein and plotting the RUeq against SH2 protein concentration. Peptide Ac-HMVMTpYAABK binds to the SHP-2 N-SH2 domain with a K_D value of 3.4 μM (Table 2). In agreement with the results from the activation experiments, truncation of the C-terminal sequence has little effect on the binding affinity. Nor does substitution of norleucine for the methionyl residues [peptide H(Nle)V(Nle)TpY] cause any significant change in the binding affinity, validating the use of norleucine as a surrogate of methionine at the randomized positions of the pY library. To test whether peptide Ac-HMVMTpY is a specific ligand for the SHP-2 N-SH2 domain, its binding affinities toward other SHP-1/-2 and Src SH2 domains were determined under identical conditions. Its affinity for the other three SH2 domains of SHP-1/-2 is at least 5-fold lower, although the four SH2 domains of SHP-1/-2 share high sequence homology and have overlapping sequence specificities toward canonical pY ligands (*8*, *13*). Its binding to Src SH2 domain is also 2−3-fold weaker. Therefore, the pY peptide selectively binds to SHP-2 N-SH2. Finally, a composite peptide (Ac-KBHMVMTpYATIL-NH2), which contains the most preferred sequences on both sides of pY, was synthesized and tested for binding to the SHP-2 N-SH2 domain. This peptide had a K_D value of 1.0 μM, only slightly lower than those of the control peptides, Ac-HMVMTpY-NH_2 (K_D = 3.2 μM) and LVpYATILNB-BKR-NH_2 (K_D = 1.9 μM). This result, together with the competition data described above, suggests that the pY peptides selected from the library (e.g., Ac-HMVMTpY-NH_2) interact with the same binding pockets on the SH2 domain surface as the canonical pY peptides (e.g., LVpYATIL), perhaps in an inverted orientation. This provides a possible explanation for their respectable binding affinities to the Src SH2 domain (Table 2), despite the fact that they do not contain an Ile at the pY+3 position. Obviously, further studies are necessary to test this notion.

An SHP-1 N-SH2 domain-binding peptide (Ac-MMRQMpY-AABK) and an SHP-2 C-SH2 domain-binding peptide (Ac-RYHGYpYAABK) were arbitrarily selected from Table 1 and tested for binding to the above SH2 domains. These pY peptides bind weakly to all of the SH2 domains (Table 2). This is not surprising, considering that no consensus sequence-(s) emerged from these selected sequences.

Identification of Positions Critical for Binding to SH2 Domain. Alanine scanning was carried out with peptide HMVMTpY to define the positions that are critical for binding to the SH2 domain. Substitution of alanine for any of the residues at positions −4 to −1 had relatively minor effect, reducing the binding affinity by ∼2-fold (Table 2). However, replacement of the histidine at the −5 position caused a 5-fold reduction in binding affinity. We reached exactly the same conclusions about the SHP-2 activation assays with the alanine scan peptides (Figure 4). Therefore, all of the N-terminal residues contribute to the overall binding energy, but the pY and −5 residues are the most critical residues.

DISCUSSION

In this work, we have demonstrated for the first time that an SH2 domain can bind specifically to pY peptides by

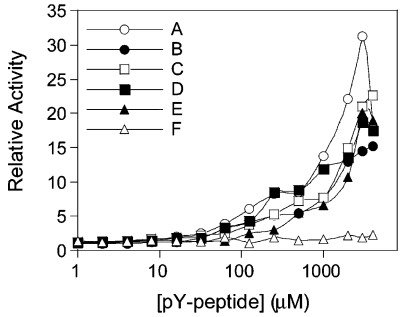

FIGURE 4: Effect of alanine substitution on the binding affinity of peptide Ac-HMVMTpY-NH_2. The peptides were tested for their ability to activate SHP-2, and the EC_{50} value for SHP-2 activation correlates with a peptide's affinity to SHP-2 N-SH2 domain. (A) Ac-HMVMTpY-NH_2; (B) Ac-HMVMApY-NH_2; (C) Ac-HMVATpY-NH_2; (D) Ac-HMAMTpY-NH_2; (E) Ac-HAVMTpY-NH_2; and (F) Ac-AMVMTpY-NH_2.

recognizing *only* the amino acid residues N-terminal to pY. The previously reported examples all involved specific interactions between the SH2 domains and residues N-terminal to the pY, *in addition to* the canonical C-terminal interactions (*7*−*9*). The PTB domains, which bind to pY peptides by recognizing only the N-terminal residues, belong to a different class of protein modules, which have entirely different protein folds (*10*). For the SHP-2 N-SH2 domain, binding with the pY peptide involves the pY residue and all five amino acid residues immediately N-terminal to pY. Removal of the phosphate group from the pY completely abolished the binding interactions, whereas substitution of alanine for any of the five N-terminal residues significantly reduced the binding affinity. Inspection of the selected sequences reveals that the SH2 domain has the most stringent side-chain requirement at positions −5, −3, and −1 (Figure 1), suggesting that these three positions and the pY are the most crucial determinants of the binding interactions. Consistent with this notion, replacement of the −5 histidine with alanine caused the largest reduction in binding affinity (>5-fold) (Table 2). Substitution of alanine for the −3 valine or −1 threonine had smaller effects (∼2-fold), probably because these are relatively conservative mutations. Note that alanine is the third most preferred amino acid at both positions (Figure 1). The overall binding affinity between peptide HMVMTpY and the SHP-2 N-SH2 domain (K_D = 3.2 μM) is comparable to those observed for the classical SHP-1/-2 SH2 domain−pY peptide interactions (e.g., K_D = 1.9 ± 0.1 μM for SHP-2 N-SH2 domain binding to peptide LVpYATIL) (Table 2).

Since the alternative binding motifs were identified from a synthetic peptide library, it is unclear at the present time whether they are physiologically relevant. Essentially all of the known SHP-2-binding proteins contain the classical ITIM motifs, [TILV]XpY[ASTVI]X[ILV] (*13*). Most of these known SHP-2 targets were identified because of the presence of ITIM motifs in their protein sequences. We performed a database search for human proteins that contain the consensus sequence [HF]XVX[TSA]Y at the Protein Information Resource (web site: http://pir.georgetown.edu/). A total of >400 "hits" were obtained, representing over 100 unique proteins (some proteins appeared multiple times under different names or fragments). It is possible that some of these proteins may prove to be bona fide binding partners of SHP-2. Some of these proteins that are likely to be involved in cell signaling are listed in Table 3. However, regardless of their physiological relevance, the alternative binding sequences should provide very useful probes for studying the cellular functions of SHP-2. For example, the pY peptides may be employed as inhibitors to specifically disrupt the association between SHP-2 and its target proteins. Because of the high sequence and structural similarities between SHP-1 and SHP-2, the classical ITIM-like pY peptides often bind to both enzymes with similar affinities (*13*). The alternative pY motifs from this work are quite selective for SHP-2 N-terminal SH2 domain (e.g., HMVMTpY) (Table 2). Understanding the structural basis for the specificity may also guide the design of peptidomimetic or small-molecule inhibitors that are specific for the N-SH2 domain of SHP-2.

Table 3: Potential SHP-2-Binding Proteins

protein	sequence motif
adenylate cyclase 2	HLVKTY
interleukin-8 receptor type A	HRVTSY
leukemia inhibitory factor receptor	HVVVSY
lysine-specific histone demethylase 1	HRVHSY
olfactory receptor	FIVVSY
protein phosphatase 1	FSVVSY
T cell activation protein phosphatase 2C	FSVLSY

In summary, this work has shown that at least some of the SH2 domains can bind to pY peptides/proteins by recognizing the sequences N-terminal to pY, in addition to the classical C-terminal binding mode. It remains to be determined whether this property is unique to the SHP-2 N-SH2 domain or more general for other SH2 domains. The structural basis for the alternative binding mode also needs to be elucidated. Such studies are already underway.

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