Determination of the Binding Specificity of the SH2 Domains of Protein Tyrosine Phosphatase SHP-1 through the Screening of a Combinatorial Phosphotyrosyl Peptide Library†

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ABSTRACT: A method for the rapid identification of high-affinity ligands to Src homology-2 (SH2) domains is reported. A phosphotyrosyl (pY) peptide library containing completely randomized residues at positions −2 to +3 relative to the pY was synthesized on TentaGel resin, with a unique peptide sequence on each resin bead (total 2.5×10^6 different sequences). The library was screened against the biotinylated N- and C-terminal SH2 domains of protein tyrosine phosphatase SHP-1, and the beads that carry high-affinity ligands of the SH2 domains were identified using an enzyme-linked assay involving a streptavidin−alkaline phosphatase conjugate. Peptide ladder sequencing of the selected beads using matrix-assisted laser desorption ionization mass spectrometry revealed consensus sequences for both SH2 domains. The N-terminal SH2 domain strongly selects for peptides with a leucine at the −2 position; at the C-terminal side of the pY residue, it can recognize two distinct classes of peptides with consensus sequences of LXpY(M/F)X(F/M) and LXpYAXL (X = any amino acid), respectively. The C-terminal SH2 domain exhibits almost exclusive selectivity for peptides of the consensus sequence, (V/I/L)XpYAX(L/V). Several representative sequences selected from the library were individually synthesized and tested for binding to the SH2 domains by surface plasmon resonance and for their ability to stimulate the catalytic activity of SHP-1. Both experiments have demonstrated that the selected peptides are capable of binding to the SH2 domains with dissociation constants (K_D) in the low micromolar range.

Signal transduction, regulation of gene expression, and other cellular processes are all mediated by two types of events: chemical modification (e.g., phosphorylation and dephosphorylation) and physical association of the proteins involved (i.e., formation of protein−protein complexes). A major advance in the latter area was the realization in 1990s that many protein−protein interactions are mediated by small protein modules, which recognize linear peptide motifs in their partner proteins (*1*). The Src homology-2 (SH2)[1] domain was among the first such modules discovered (*2*). It consists of ∼100 amino acids and is an independently folded functional module found in a wide variety of signaling proteins (*3*, *4*). SH2 domains bind to their interacting proteins by recognition of linear phosphotyrosine (pY)-containing sequence motifs (*5*, *6*). Structural studies of SH2-pY peptide complexes reveal that 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 forms a bidentate interaction with the pY phosphate group (*7*−*9*). 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. Presumably, the binding specificity of different SH2 domains directs the proteins that contain them to different pY receptors, thereby transducing specific signals to downstream proteins. An important task in signal transduction research is therefore to determine the binding specificities of these SH2 domains as well as other protein modules.

Several combinatorial library methods have been devised to systematically determine the binding specificity of SH2 domains. The first method employed an SH2 affinity column to enrich the SH2-binding sequences from a pY peptide library. Sequencing of this enriched pool generated a consensus sequence(s) based on preferentially selected amino acid(s) at a given position (*5*, *6*). The second method involved screening support-bound libraries against a fluorescently labeled SH2 domain (*10*). The positive beads with the bound SH2 were removed from the library by fluorescence-activated bead sorting and sequenced as a pool. Both methods provide information on the consensus sequence but do not give individual sequences. As several amino acids are often enriched at a given position, there is still a great deal of uncertainty as to which particular amino acid combination

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[1] Abbreviations: Nle, norleucine; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; SH2, Src homology 2; SH2(N), N-terminal SH2 domain; SH2(C), C-terminal SH2 domain; pY, phosphotyrosine; MALDI MS, matrix-assisted laser desorption ionization mass spectrometry; MBP, maltose-binding protein; GST, glutathione-*S*-transferase.

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will produce a high-affinity binder. It is possible that a peptide featuring the most preferred residues at each position actually does not have the highest affinity. Furthermore, because these methods select for *both* affinity and abundance of certain types of sequences, a high-affinity peptide that is present only in minute amounts in the library may be entirely missed. In the third method, bacteriophage bearing short random peptide sequences on their surfaces was phosphorylated by a kinase cocktail and selected against an immobilized SH2 domain (*11*). The sequences of the SH2-binding pY peptides were determined by amplifying the bound phage and sequencing their DNA. The limitation of this method is that the starting library is grossly biased by the specificities of the kinases (only 2−3% phage were phosphorylated). Finally, a method has been developed to combine library affinity selection with mass spectrometry to identify SH2-binding sequences (*12*). However, this method has only been demonstrated with very small libraries (361 members).

We report here yet another method to identify specific binding motifs for SH2 domains or other protein modules from a combinatorial peptide library. The library (up to 2.5 $\times$ 10^6 individual sequences) was synthesized on beads ($\sim$90 μm in diameter) using the split-pool method (*13*, *14*), with each bead carrying a unique peptide sequence. The library was screened for SH2 binding using an enzyme-linked assay, and the positive beads were manually removed from the library and sequenced by matrix-assisted laser desorption ionization (MALDI) mass spectrometry. This method provides both individual sequences and a consensus sequence(s) and should be readily applicable to other protein modules. The power of this method has been demonstrated by the rapid identification of high-affinity pY peptide ligands for the SH2 domains of protein tyrosine phosphatase SHP-1 (*15*−*18*).

MATERIALS AND METHODS

Materials and General Methods. 5-Bromo-4-chloro-indolyl-phosphate (BCIP), biotin, and streptavidin-alkaline phosphatase conjugate were purchased from Sigma Chemical Co. Gelatin was purchased from Bio-Rad. All peptide synthesis reagents and resins were purchased from Advanced ChemTech (Louisville, KY). Soluble peptides including biotinylated peptides were synthesized on Wang resin using standard Fmoc chemistry on 0.015−0.2 mmol scale as detailed previously (*19*). Phosphotyrosyl (pY) peptides were synthesized using unprotected *N*-Fmoc-pY during the coupling reactions (*20*). Crude peptides of $\geq$80% purity (as judged by analytical HPLC) were used directly, whereas less pure samples 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. SHP-1, its isolated N- and C-terminal SH2 domains, and their maltose-binding protein (MBP) or glutathione-S-transferase (GST) fusion proteins were expressed and purified as previously described (*21*−*23*). Biotinylation of proteins was carried out by treating MBP−SH2(N), MBP, or the free SH2(C) protein (180−800 μM in 50 mM sodium phosphate, pH 7.0, 150 mM NaCl) with 1.8−2.5 M equiv of N-hydroxysuccinimidobiotin for 30 min at room temperature. The samples were 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 protein was collected, concentrated in a Centriprep-10 concentrator, and quickly frozen in the presence of 33% glycerol.

Library Construction. The pY peptide library, Ac-DEXX-pYXXXIBBRM (X = Nle or any natural amino acid except for Met and Cys), was synthesized on 5 g of TentaGel S NH_2 resin (80−100 μm, 0.3 mmol/g) as previously described (*24*). The random positions were generated by the split-pool synthesis method (*13*, *14*) and the coupling reactions were carried out with 5 equiv of reagents for 3−4 h and repeated once. To generate some chain-termination products for peptide ladder sequencing by MALDI mass spectrometry, a small amount of capping reagents was added to the coupling reactions (along with the individual amino acids) during the synthesis of the randomized region (*25*). *N*-Acetylalanine (10%) was used for Nle; a mixture of *N*-acetylalanine and *N*-acetylglycine (5% each) was used for Gln and Ile; and *N*-acetylglycine (10%) was used for all other amino acids. Side-chain deprotection of the resin-bound peptides was performed as previously described (*24*).

Library Screening and MALDI Peptide Sequencing. Library beads were exhaustively washed with CH_2Cl_2, DMF, H_2O, and TBS buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) containing 0.1% (w/v) gelatin. The library beads were incubated for 1 h in the above buffer to block any nonspecific protein binding sites. The beads were next suspended in fresh TBS buffer containing 1.0 μM biotinylated MBP−SH2(N) or 1.5−3.5 μM biotinylated SH2-(C) and the mixture was incubated for 6−12 h at 25 °C with constant mixing. After initial incubation with SH2 protein, the mixture was supplemented with 10−14.3 nM streptavidin-alkaline phosphatase conjugate and 25 mM sodium phosphate. The mixture was shaken at room temperature for 15 min and quickly washed with PBS buffer (20 mM sodium phosphate, pH 7.4, 150 mM NaCl) containing 0.1% Tween 20 (2 $\times$ 3 mL), 3 mL of PBS buffer, and 3 mL of TBS buffer (20 mM Tris-HCl, pH 8.5, 150 mM NaCl). The resin beads were suspended in 12 mL of the TBS buffer (pH 8.5) and incubated in the presence of 1.4 mM 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). Typically, the staining reaction was allowed to proceed for 30−60 min at room temperature before being quenched by incubation in 6 M guanidine hydrochloride for 15−20 min. The beads were washed exhaustively with water prior to sequence analysis. Positive beads were readily identified by their intense turquoise color and were manually removed from the library with a micropipet under a low-power microscope. A control screening was performed with biotinylated MBP (no SH2) under the same conditions but resulted in no colored beads. Individually selected beads were each incubated with 20 μL of 20 mg/mL CNBr in 70% formic acid for 20−24 h in the dark. The solvent and excess CNBr were removed under vacuum and the released peptides from each bead were dissolved in 3 μL of 0.1% TFA in water. Sequence analysis was carried out on a Kratos Kompact MALDI−III mass spectrometer in the positive ion mode as described previously (*24*).

SH2-pY Peptide Binding Assays. Two different assays were performed to detect binding of SH2 domains to pY peptides selected from the library. In method A, pY peptides were assayed for their ability to stimulate SHP-1 activity (*22*, *23*). The assay reaction (total volume of 50 μL) contained SHP-1 (73 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 0−600 μM pY peptide. The reaction was allowed to proceed for 31 min at room temperature before being quenched with 950 μL of 1 M NaOH, and the absorbance at 405 nm was measured on a UV−vis spectrophotometer. Peptide concentrations were determined by hydrolysis of pY to completion with alkaline phosphatase followed by the malachite green assay of the released inorganic phosphate (*26*). All reactions were carried out in triplicates.

In method B, the Pharmacia BIAcore was employed (*27*). pY peptides were biotinylated at their N-termini via solid-phase peptide synthesis and immobilized onto streptavidin-coated sensorchips by passing a pulse of 0.1 μM peptide solution in HBS buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, and 3.4 mM EDTA) over the surface of a chip. For most peptides, 1−5 μL at a flow rate of 10 μL/min was sufficient to result in 150−350 response units (RU). The amount of immobilized pY peptide was determined by saturating the surface with GST-SH2(N+C) and measuring the maximal response units. This low loading density of pY peptides drastically reduces the avidity effects due to GST dimerization (*28*). Data for secondary plot analysis was generated by passing increasing concentrations (0−50 μM) of an SH2 protein in HBS buffer over the sensor chip at 10 μL/min for 3 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 domain concentration. The data were fit to the equation

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

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

RESULTS

Library Design, Construction, and Screening. The majority of SH2 domains recognize primarily the pY residue and the three residues immediately C-terminal to pY (*5*, *6*), although it has been reported that, for a few SH2 domains including those of SHP-1, the −2 position (relative to pY) of a pY peptide is also important for high-affinity interaction (*29*, *30*). Thus, we designed a pY library, acetyl-DEXXpYXXXI-BBRM-resin, in which the two residues immediately N-terminal to the pY and the three C-terminal residues are randomized [X = norleucine (Nle) or any of the 18 natural amino acids except for Met and Cys; B = β-alanine]. 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 (*25*). Two acidic residues, Asp and Glu, were added at the N-terminus to ensure reasonable solubility of all peptides. The two β-alanines add some flexibility to the peptides, making them more accessible to a protein target (*31*). An invariant Ile is placed at the +4 position because our earlier screening of a tetrapeptide library, Ac-LKpYXXXXBBRM-resin, indicated that the SH2(N) of SHP-1 has very weak selectivity for a hydrophobic residue at this position (unpublished results). Methionine is excluded from the randomized region to avoid internal cleavage during CNBr treatment and is replaced by the isosteric norleucine residue. In all of our studies so far (*24*, *32*), we have found no significant effect on either protein binding or enzyme catalysis when Nle is substituted for Met. The library was synthesized on TentaGel S NH_2 resin (2.86×10^6 beads/g) using the split-pool method (*13*, *14*), 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. Partial chain termination was effected by the addition of 10% *N*-acetylglycine and/or *N*-acetylalanine to the individual coupling reactions during the construction of the randomized region (see Experimental Procedures).

The library (theoretical diversity = 19^5 or 2.5×10^6) was screened for binding to both SH2 domains of SHP-1. For the N-terminal SH2 domain, a maltose binding protein fusion, MBP−SH2(N), was employed, whereas the free C-terminal SH2 domain was used. Both SH2 domains were biotinylated on a surface lysine residue(s) by treatment with *N*-hydroxysuccinimidobiotin. Screening was based on the assumption that binding of the biotinylated SH2 domain to a bead that carries a high-affinity pY peptide for the SH2 domain should recruit a streptavidin-alkaline phosphatase conjugate to the surface of that bead. Upon the addition of 5-bromo-4-chloro-3-indolyl phosphate (BCIP), the bound alkaline phosphatase would cleave BCIP into an indole, which should instantaneously dimerize 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.

Analysis of Selected Sequences. Screening of 1.0 g of resin (~2.86×10^6 beads) each against the N- and C-terminal SH2 domains of SHP-1 resulted in ~150 and ~300 colored beads, respectively. Peptide ladder sequencing (*24*, *25*) was performed for all 150 beads selected against MBP−SH2(N) to give 97 unambiguous sequences (Table 1). The rest of the beads (total 53) produced mass spectra that missed one or a few peaks in the peptide ladder and, therefore, complete sequence assignment was not possible. Out of the 300 beads selected against the SH2(C) domain, ~175 of the brightest color were isolated from the library and individually sequenced to give 112 unambiguous sequences (Table 1).

Analysis of the selected sequences reveals that each SH2 domain exhibits two different consensus sequences and the two SH2 domains have overlapping but distinctive specificities (Table 1 and Figure 1). For the N-terminal SH2 domain, the majority of the selected sequences (85 of 97) belong to class I, which has a consensus sequence of L(Y/H)pY(M/F)X(F/M). An earlier screening of the tetrapeptide library Ac-LKpYXXXXBBRM yielded the same consensus, demonstrating the reproducibility of the library method (data not shown). A striking feature of this SH2 domain is that the strongest selectivity is at the −2 position, with a leucine being the most frequently observed residue; even norleucine and isoleucine, which are structurally similar to leucine, are only found sparingly at this position. The SH2 domain also exhibits strong selectivity for residues C-terminal to the pY.

Table 1: Sequences of Peptides Selected from the pY Library[a]

SH2(N)

Class I	LHpYMFM	LHpYMFM	MHpYMLF	**ClassII**
LYpYFAF	LHpYMFA	LQpYMTF	IRpYFSF	LNpYAFL
LLpYFMY	LHpYMAF	MQpYMLF	IHpYMYM	LNpYAMF
LYpYFNY	LHpYMVF	LNpYMEL	LHpYMVL	LNpYAFL
LLpYMFM	LHpYMVL	LNpYMLF	IHpYMVF	IYpYANL*
LYpYMNM	LHpYMLF	LNpYMYF	LWpYLNM	LYpYANL*
LYpYMDM	LHpYYYF	LNpYMAM	MLpYLAL	LYpYAAL
LYpYMQL	LHpYFLM	LNpYMNF	TDpYNWV	LYpYADL
IYpYMQM	LHpYLQM	LNpYMIF	KApYYPF	LHpYAIF
IYpYMNL	LHpYLMM	LEpYMMF	NHpYFMR	LApYAWL
LYpYMNV	PRpYMAF	IQpYMFM	SYpYYLR	LWpYAQL
LYpYMRY	LKpYMRF	LNpYVLM		NHpYADL
LYpYMSL	LRpYMRM	VQpYLYF		MHpYVLL
LYpYMFP	LKpYMAF	LNpYFAF		
LYpYMRF*	LKpYMSF	LQpYFMM		
LYpYMAY	VKpYMLF	LSpYFFM		
LYpYMND	MKpYMRF	ISpYFLF		
LYpYMLE	MRpYFRL	NYpYMMQ		
LYpYMFQ	LKpYFFW	MHpYFMY		
LYpYLAP	LKpYFMH	MHpYFVY		
LYpYLFF	LRpYYMF	MHpYFMF		
PYpYMRM	VKpYFMF	MHpYFMF		
LHpYMDF	LKpYLMF	MHpYMYY		
LHpYMYY	LRpYIVM	MHpYLYF		
LHpYMLM	LQpYMLF	MHpYMLM		

SH2(C)

Class I	IVpYAQM	**ClassII**	HGpYYMK	FVpYYMK
TTpYARL	MQpYAMI	MWpYYAR	RWpYYMK	IQpYYMK*
VHpYARL*	YQpYAYL	LWpYYMQ	KNpYYMK	PRpYYMR
IApYARL	HHpYAVL	AWpYYMQ	HKpYYMK	KRpYYMR
HRpYARL	SHpYAVL	YKpYYMQ	HSpYYMK	DTpYYMR
VMpYARL	YQpYAIV	YPpYYMQ	NHpYYMK	GFpYYMR
YRpYARL	YNpYAIV	NGpYYMQ	SSpYYMK	YYpYYMR
QGpYARL	FRpYAIL	GSpYYMQ	AEpYYMR	KYpYYMR
TRpYARL	YNpYALL	FWpYYML	TEpYYMR	NYpYYMR
RWpYARL	FHpYAIL	QQpYYML	MApYYMR	WGpYYMR
VTpYARN	LWpYALL	LGpYYML	RHpYFMR	TGpYYMR
YRpYARI	HHpYALL	SDpYFMR	KHpYFMR	QNpYYMR
LNpYARV	VKpYALL	TNpYYMK	YHpYFMK	QIpYYMR
IHpYAKV	LYpYALL	VNpYYMK	NHpYFMR	VGpYYMR
IVpYAKL	PVpYALL	DIpYYMK	TWpYFMR	LPpYYMR
LYpYAKL	HFpYAAV	NEpYYMK	KTpYFMR	VSpYYMR
RRpYAKV	VYpYAAL	QQpYYMK	YApYYMK	ETpYYMR
PYpYAKV	LWpYSLV	EDpYYMK	PApYYMK	IEpYYMR
LYpYAHI	VHpYMKL	GDpYYMK	VSpYYMK	QWpYFMR
IVpYAEL	IQpYMRL	REpYYMK	GSpYYMK	AWpYFMR
IQpYAEL	TEpYVKV	EHpYYMK	PSpYYMK	VSpYFMR*
VEpYAEL		RKpYYMN	DGpYYMK	VSpYFMR
VIpYADL		RApYYMK	SWpYYMK	DTpYFMK
VIpYANL				

[a] (*) Peptides selected for testing. M, norleucine.

At position +1, norleucine is most preferred, followed by phenylalanine and leucine, whereas at position +3, phenylalanine is the most preferred residue followed by norleucine, leucine, and tyrosine (Figure 1). Some specificity is also observed at position −1, where tyrosine and histidine are frequently selected. There is little selectivity at the +2 position. A small number of the selected sequences (12 of 97) clearly belong to a different class, with a consensus sequence of LXpYAXL (class II) (Table 1). Like in class I peptides, a leucine is the most frequently selected residue at the −2 position. On the C-terminal side, alanine and leucine are almost invariant at the +1 and +3 positions, respectively.

The C-terminal SH2 domain also selected two distinct classes of sequences (Table 1). Class I peptides (total 44) exhibit a consensus of (V/I/L)XpYAX(L/V), in which alanine is almost exclusively selected at the +1 position, whereas leucine is the most preferred amino acid at the +3 position (Figure 1). This SH2 domain also shows selectivity at other positions. It prefers a hydrophobic residue (e.g., valine, isoleucine, leucine, or tyrosine) at position −2, a hydrophilic residue (e.g., histidine, arginine, or glutamine) at position −1, and a positively charged residue (e.g., arginine or lysine) at position +2. The class II peptides (total 68) have a consensus sequence of XXpYYM(K/R) (Table 1). In contrast to class I sequences, the most selective position is actually at position +2, where norleucine is exclusively selected. A tyrosine is strongly preferred at position +1, with phenylalanine being the only acceptable substitution. A positively charged residue, lysine or arginine, is highly preferred at position +3. Essentially all of the selected sequences have the consensus sequence at the C-terminal side; they only differ by their N-terminal sequences. Note that the class I sequences of the C-terminal SH2 domain closely resemble the class II sequences of the N-terminal SH2 domain.

Affinity Measurements of Selected Sequences. Several representative peptides selected from the two libraries were re-synthesized individually and tested for binding to the SH2 domains of SHP-1 using the surface plasmon resonance technique (BIAcore) (Table 2). Immobilization of the pY peptides onto streptavidin-coated sensorchips was effected by the addition of a biotin to the N-termini of these peptides during solid-phase synthesis. To maximize the accessibility of the surface bound peptides to an incoming SH2 domain, a flexible linker of Ala-β-Ala-β-Ala-β-Ala (ABBB) is added between the biotin and the N-terminus of a pY peptide (e.g., biotin-ABBBIYpYANLI). Initially, we attempted to titrate the surface directly with MBP-SH2(N) as carried out in our previous work (*22*, *23*). Although we obtained good-quality data for some of the pY peptides (data not shown), nonspecific binding of MBP to the chip surface became a problem, particularly at high concentrations (e.g., 50 μM). Therefore, we performed all of the binding experiments with glutathione-*S*-transferase fusion proteins (GST-SH2), which had much less nonspecific binding to the chip surface under our assay conditions. However, GST is known to dimerize, resulting in the measurement of avidity instead of affinity (*28*). To minimize the avidity effect, we used the streptavidin-coated sensorchip that avoids overloading the surface with pY peptides. We also used two known ligands of SHP-1 SH2 domains for comparison: peptide LKpYLYLV of erythropoietin receptor (EpoR) known to bind to the N-terminal SH2 domain of SHP-1 (K_D = 1.8 μM for Ac-PHLKpYLYLV-VSDK) (*22*, *33*) and peptide ITpYSLLK of B cell Fc receptor known to bind to the C-terminal SH2 domain (K_D = 2.8 μM for EAENTITpYSLLKH) (*23*, *34*). Our data in Table 2 show a reasonable agreement between the K_D values determined with GST-SH2 proteins in this work and the literature values obtained with MBP−SH2 proteins (*22*, *23*).

Figure 2A shows the BIAcore sensograms for the binding of GST-SH2(N) to immobilized peptide IYpYANLI, a class II peptide selected against the N-terminal SH2 domain; flow of increasing concentrations of the SH2 protein (0.4−25 μM) over the chip resulted in increasing and eventually saturating equilibrium response units (RU_{eq} = response at 12 s after the end of injection). Plot of the RU_{eq} values against SH2 concentration clearly showed saturation behavior and data fitting gave an equilibrium dissociation constant (K_D) of 0.60 μM (Figure 2B). This peptide also binds to the C-terminal SH2 domain, although with slightly lower affinity (K_D = 1.4 μM) (Table 2), consistent with the observed overlapping specificity of the two SH2 domains. Likewise, another class II peptide selected for the N-terminal SH2 domain, LYpY-

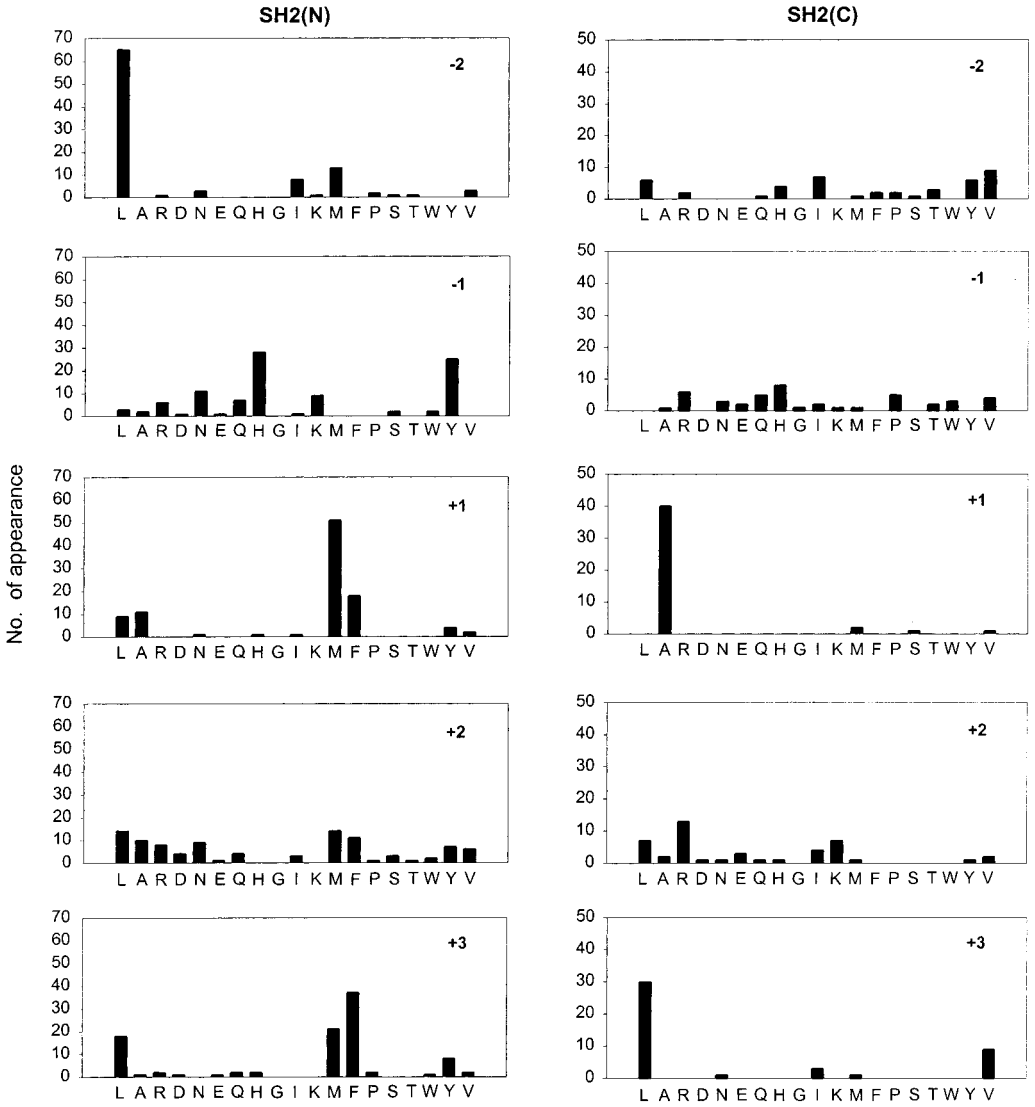

FIGURE 1: Specificity of the N- and C-terminal SH2 domains of SHP-1. Displayed are the amino acids identified at each position from −2 to +3 relative to pY (position 0). Number of appearance on the *y* axis represents the number of selected sequences that contain a particular amino acid at a certain position [out of a total of 97 for the N-SH2 domain and 44 (class I) for the C-SH2 domain]. M, norleucine.

Table 2: Dissociation Constants (K_D, μM) of Selected Peptides

peptide[a]	GST-SH2(C)	GST-SH2(N)
LKpYMQMF	ND	2.4 ± 0.4
LKpYLYLV	ND	2.7 ± 0.4
VHpYARLI	1.8 ± 0.2	4.9 ± 0.7
ITpYSLLK	1.2 ± 0.2	ND
LYpYANLI	2.0 ± 0.1	0.65 ± 0.05
IYpYANLI	1.4 ± 0.1	0.60 ± 0.06
VYpYANLI	1.9 ± 0.1	1.1 ± 0.1
AYpYANLI	>50	>50

[a] Biotin-ABBB (B = β-Ala) was attached to the N-terminus of each peptide. ND, not determined; M, methionine.

ANLI, binds to the N- and C-terminal SH2 domains with K_D values of 0.65 and 2.0 μM, respectively. Peptide VHpYARLI, which was selected for the C-terminal SH2 domain, also binds both SH2 domains but with slightly higher affinity for the C-terminal SH2 domain (K_D = 4.9 and 1.8 μM for the N- and C-terminal SH2 domains, respectively). A class I peptide of the N-terminal SH2 domain, LKpYMQMF (M, methionine) (which was selected from the initial tetrapeptide library), binds to the N-terminal SH2 domain with a K_D value of 2.4 μM. Thus, all of the selected peptides we have tested bind to SHP-1 SH2 domains with similar affinity to the pY peptides derived from known SHP-1 partner proteins. We have not been able to demonstrate specific binding of the C-terminal SH2 domain to the class II peptides (e.g., IQpYYMKI, IQpYY(Nle)KI, VSpYYMRI) by this technique.

Stimulation of SHP-1 Activity by pY Peptides. To further characterize the selected pY sequences, we have examined their ability to stimulate the catalytic activity of SHP-1. It has previously been established that the N-terminal SH2 domain of SHP-1 autoinhibits its phosphatase domain by forming a noncovalent intramolecular SH2−PTP complex (*22*). Binding of a pY peptide to the N-terminal SH2 domain disengages the intramolecular complex and stimulates the enzymatic activity by ~30-fold (*22*, *23*). 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. For fair comparison, we synthesized a shorter version of EpoR pY429 peptide, LKpYLYLV, as our benchmark. As shown in Figure 3A, all of the peptides selected against the

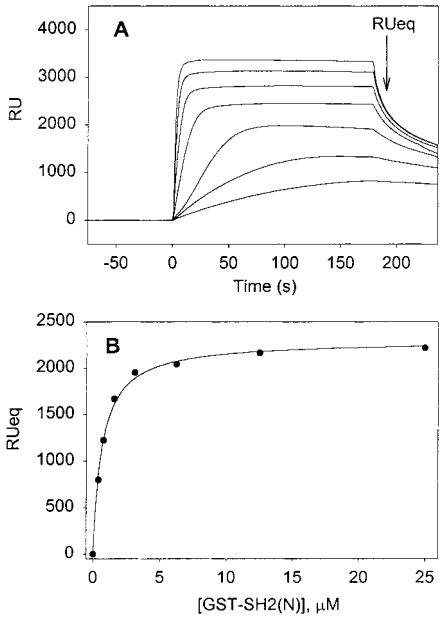

FIGURE 2: BIAcore analysis of the binding of GST-SH2(N) to peptide IYpYANLI. (A) Overlaid sensograms at increasing concentrations (0.39, 0.78, 1.56, 3.12, 6.25, 12.5, and 25 μM) of GST-SH2(N). (B) Plot of resonance signal under the equilibrium binding conditions (12 s after the end of injection) against SH2 concentration. Data were fitted to equation: $RU_{eq} = RU_{max} \times [SH2]/(K_D + [SH2])$.

N-terminal SH2 domain exhibited potent stimulation of SHP-1 in a concentration-dependent manner. In fact, the selected peptides are more potent than the EpoR peptide. These results again suggest that the selected pY peptides are capable of binding to the N-terminal SH2 domain. We have avoided making any quantitative comparison in the binding affinities of these peptides based on the stimulation data, the magnitude of which is complicated by the differential ability of these peptides to inhibit the phosphatase activity during the stimulation assays (through competition with the assay substrate *p*-nitrophenyl phosphate).

Importance of the −2 and +5 Positions. Since the N-terminal SH2 domain strongly selects for a leucine at the −2 position, we examined the importance of the −2 residue in binding to the SH2 domain by replacing the leucine in LYpYANLI with an isoleucine, valine, or alanine. Among the resulting peptides, IYpYANLI (which is one of the selected class II peptides) has essentially the same binding affinity to the parent peptide (K_D = 0.60 vs 0.65 μM) (Table 2). Substitution of a valine for the leucine residue resulted in a 2-fold reduction in binding affinity (K_D = 1.1 μM for VYpYANLI). However, substitution of an alanine at the −2 position drastically reduced the binding affinity (K_D > 50 μM for AYpYANLI). Consistent with the BIAcore results, peptides LYpYANLI, IYpYANLI, and VYpYANLI exhibited similar potencies in stimulating SHP-1, whereas peptide AYpYANLI was >10-fold less active (Figure 3B). The effect of a Leu→Ala mutation at the −2 position was even greater for the class I peptides. For example, peptide AYpYMRFI was nearly 2 orders of magnitude less potent in stimulating SHP-1 than LYpYMRFI (Figure 3B). These results demonstrate the importance of the −2 residue in binding to the N-terminal SH2 domain and validate the strong selection for a leucine (or norleucine and isoleucine) during library screening. It should be noted that a control peptide (not

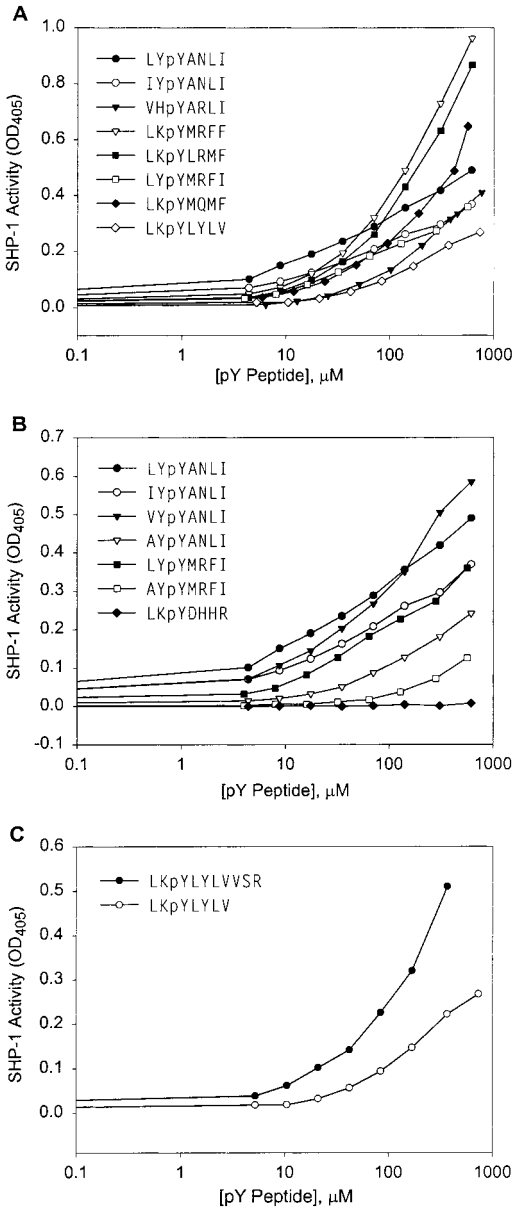

FIGURE 3: Activation of SHP-1 by various pY peptides. (A) Comparison of several selected peptides with the EpoR pY429 peptide (LKpYLYLV). Peptides not shown in Table 1 were selected from the tetrapeptide library LKpYXXXX. (B) Effect of the −2 residue on SH2(N) binding. (C) Effect of residues beyond +4 position on SH2(N) binding. M, methionine.

selected from the library), LKpYDHHR, resulted in no stimulation of SHP-1 at concentrations up to 500 μM (Figure 3B). This result suggests that the pY residue and a leucine at position −2 are necessary but not sufficient for high-affinity binding to the N-terminal SH2 domain of SHP-1.

The contribution of the −2 residue for binding to the C-terminal SH2 domain was also examined. While peptides VHpYARLI, LYpYANLI, IYpYANLI, and VYpYANLI all bind the SH2 domain with very similar K_D values (~2 μM), peptide AYpYANLI showed much weaker affinity (K_D > 50 μM) (Table 2). This demonstrates that the C-terminal SH2 domain also requires a hydrophobic residue at the −2 position for high-affinity binding, although the precise nature of the side chain is less critical than for the N-terminal SH2 domain. Note that 80% of the class I sequences selected against the C-terminal SH2 domain contained valine, iso-

leucine, leucine, tyrosine, threonine, proline, or phenylalanine at the −2 position, whereas none of the sequences contained alanine, aspartic acid, asparagine, glutamic acid, glycine, or lysine at this position (Table 1 and Figure 1).

We have noted that the residues beyond the +4 position may also contribute to the overall binding affinity, at least for the binding of some peptides to the N-terminal SH2 domain of SHP-1. There was a ~5-fold reduction in the stimulatory activity when the EpoR pY429 peptide Ac-LKpY-LYLVVSR was shortened to Ac-LKpYLYLV (Figure 3C).

DISCUSSION

The dual SH2 domain-containing phosphatase SHP-1 and its close relative SHP-2 belong to an intriguing subfamily of nontransmembrane protein tyrosine phosphatases. Both SHP-1 and SHP-2 have been shown to actively participate in many signaling pathways, acting both as positive and negative regulators (reviewed in ref *35*). Their SH2 domains play essential roles in these signaling events by binding to and thereby recruiting the catalytic activity to different tyrosyl phosphorylated receptors. Although the pY motifs on the receptors that are responsible for SHP-1 and SHP-2 binding have been determined in many cases, the precise mode of interaction (e.g., which SH2 is responsible for binding?) is less clear. In this work, we have carried out a systematic evaluation of the binding specificity for both SH2 domains of SHP-1. Such information will greatly facilitate the identification of the cognate binding site(s) on the known receptors for each SH2 domain of SHP-1 as well as new target proteins of SHP-1.

A surprising finding of this work is that, for the N-terminal SH2 domain, the −2 position is most critical for high-affinity binding. Of the 97 sequences determined, 65 (67%) contained a leucine at position −2; the rest of the sequences contained the highly homologous norleucine, isoleucine, or valine at this position (Figure 1). Substitution of an alanine for the −2 leucine resulted in very poor binding sequences (K_D > 50 μM for AYpYMRFI and AYpYANLI) (Figure 3B and Table 2). On the basis of the screening results (Figure 1), the BIAcore binding data (Table 2), and literature data (vide infra), we propose the following order of preference at the −2 position by the N-terminal SH2 domain of SHP-1: leucine, isoleucine, norleucine > valine ≫ other amino acids. The C-terminal SH2 domain also exhibits strong preference for a hydrophobic residue at the −2 position, although the precise nature of the side chain is less critical than the N-terminal SH2 domain. Data from this work as well as literature reports suggest the following order of preference: valine, isoleucine, leucine > norleucine, tyrosine, threonine, serine, histidine, proline, phenylalanine ≫ alanine or hydrophilic amino acids. The importance of the −2 residue for binding to the SH2 domains of SHP-1 and SHP-2 has previously been noted by others, through comparison of their recognition pY motifs on various receptors and mutagenesis studies (*29*, *30*, *36*). Our current results are in excellent agreement with these literature data. Since most SH2 domains of known specificity show either no or little selectivity on the N-terminal side of pY (*5*, *6*), the SH2 domains of SHP-1 and SHP-2 represent a novel class of SH2 domains whose binding specificity is governed by both N- and C-terminal residues.

Another interesting finding is that the N-terminal SH2 domain of SHP-1 is capable of recognizing peptides of two distinct consensus sequences on the C-terminal side of pY [LXpY(M/F)X(F/M) vs LXpYAXL]. The selected peptides from both classes bind to the N-terminal SH2 domain with similar affinity and effectively stimulate the catalytic activity of SHP-1. Presumably, the two types of sequences bind to the N-terminal SH2 domain in different modes. It is worth noting that among the class I sequences, norleucine and phenylalanine are interchangeable at the +1 and +3 positions (i.e., pYMXF, pYMXM, pYFXM, and pYFXF were all selected by the SH2 domain), although there is slight preference for norleucine at the +1 position and phenylalanine at the +3 position. We have previously found through library screening that for peptide deformylase, whose physiological substrates are *N*-formylmethionyl peptides, *N*-formylnorleucyl, and *N*-formylphenylalanyl peptides are the only alternative substrates of significant deformylation reaction (*24*). The X-ray crystal structure of a deformylase-inhibitor complex showed that the *n*-butyl side chain of norleucine adopts a bent conformation that effectively mimics the phenyl ring of a phenylalanine (*37*).

The C-terminal SH2 domain also selected peptides of two distinct consensus sequences, (V/I/L)XpYAX(L/V) (class I) and XXpY(Y/F)M(K/R) (class II). Interestingly, the class I consensus sequence, (V/I/L)XpYAX(L/V), overlaps with the class II consensus sequence of the N-terminal SH2 domain (LXpYAXL). However, due to its narrower specificity at positions −2 and +3, the N-terminal SH2 domain will likely bind only a subset of the SH2(C)-binding sequences (see below for more discussion). The reason for the selection of class II sequences for SH2(C) is not yet clear. We have not yet been able to demonstrate their specific binding to the C-terminal SH2 domain by surface plasmon resonance (SPR). However, they are unlikely a result of nonspecific binding, as they did not show up during library selection against the N-terminal SH2 domain or control selections (no SH2 domain). Since free SH2(C) was used in selection, these sequences may have been selected against a population of biotinylated SH2 domain whose pY peptide-binding site was perturbed by biotin modification. There is also a remote possibility that the SH2 domain binds to these peptides in a manner that does not trigger significant SPR signal change. These possibilities are currently under investigation.

Our library results are in agreement with the literature data on the recognition motifs of SHP-1. Table 3 lists some of the pY motifs found in various receptors that have been implicated in SHP-1 binding. These motifs clearly show a consensus sequence of (V/I/T/L)XpYAX(L/V) (Figure 4). At the +1 position, alanine is most frequently observed; the sterically similar serine and, much less frequently, threonine and valine are the only allowed substitutions at this position, in excellent agreement with the library selection results (Figure 1). For the majority of these pY motifs, it is not yet clear which of the SH2 domains of SHP-1 is responsible for binding. On the basis of our library data, we predict that all of the peptides in Table 3 should be capable of binding the C-terminal SH2 domain, with the exception of the pY motifs from EpoR (LKpYLYL) (*33*) and IL-3 receptor (LEpYLCL) (*38*), which belong to the class I sequences of the N-terminal SH2 domain and are indeed known to bind to the N-terminal SH2 domain (*22*, *33*). We further predict that the subset of sequences in Table 3 that contain a leucine or isoleucine at the −2 position [e.g., ITpYSLL of FcγRIIB, IHpYSEL of

Table 3: Reported SHP-1 Binding Sites

Protein	Recognition motif	Ref.
PIRB	VTpYAQL	48
	SVpYATL	
	SLpYASV	
MAFA	SIpYSTL[a]	49
FcγRIIB	ITpYSLL[a,b]	34
CD22	IHpYSEL[b]	41, 42
	VSpYAIL	
	VDpYVTL	
PECAM	VQpYTEV	50
	TVpYSEV	
IL-3R	LEpYLCL[b]	38
CD33	LHpYASL[a,b]	40
	TEpYSEV[a]	
ILT2	VTpYAQL	51
	SIpYATL	
ILT3	VTpYAQL	52
	SVpYATL	
	VTpYAKV	
gp49B1	IVpYAQV	53
	VTpYAQL	
PILRα	IVpYASL[a,b]	39
	TLpYSVL[a]	
LAIR-1	VTpYAQL	54
	ITpYAAV	
SHPS-1	ITpYADL	55
	LTpYADL	
	TEpYASI	
	SEpYASV	
C-CAM	VApYTVL	56
	TVpYSEV	
EpoR	LKpYLYL[b]	33
KIR	VTpYAQL[a,b]	30, 43

[a] Sequences reported to bind the SH2(C) domain. [b] Sequences reported to bind to the SH2(N) domain.

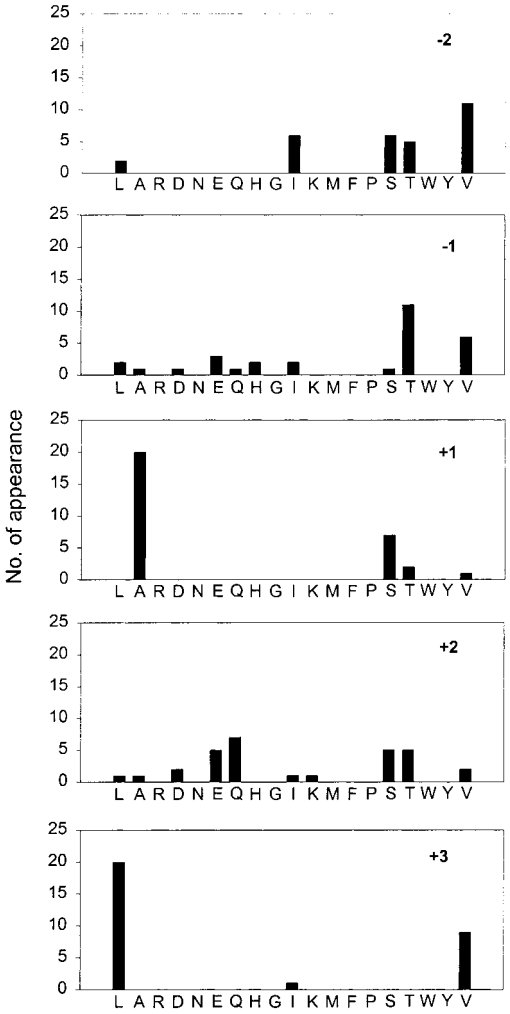

FIGURE 4: Analysis of the pY motifs from receptors that are known to bind SHP-1. Displayed are the amino acids identified at each position from −2 to +3 relative to pY (position 0). Number of appearance on the *y* axis represents the number of known and putative SHP-1 binding sequences that contain a particular amino acid at a certain position (out of a total of 30 listed in Table 4).

CD22, LHpYASL of CD33, IVpYASL of PILRα, IVpYAQV of gp49B1, ITpYAAV of LAIR-1, and (L/I)TpYADL of SHPS-1] and, perhaps with lower affinity, the motifs with a valine at position −2 (e.g., VTpYAQL) should also be capable of binding the N-terminal SH2 domain. Our predictions have turned out to be correct in all cases for which binding data are available. For example, peptides ITpYSLL and IVpYASL, the only peptides whose K_D values have been determined, bind effectively to both SH2 domains of SHP-1 (*23*, *34*, *39*). Peptide LHpYASL of CD33 has also been reported to bind to both SH2 domains of SHP-1 (*40*). Peptide IHpYSEL of CD22 is known to bind the N-terminal SH2 and stimulate SHP-1 activity (no data yet available for its interaction with the C-terminal SH2 domain) (*30*, *41*, *42*). The killer cell inhibitor receptor (KIR) motif, VTpYAQL, showed detectable binding with the isolated SH2(C) domain but not with the SH2(N) domain under similar conditions (*43*). However, this peptide exhibits potent stimulation of SHP-1 activity in a concentration dependent manner (*30*), indicating that it is capable of binding to the N-terminal SH2 domain at higher peptide concentrations. This is in keeping with our prediction that pYAXL peptides containing a valine at position −2 will bind the C-terminal SH2 domain more effectively than the N-terminal SH2 domain of SHP-1. Peptide TLpYSVL derived from PILRα, which does not have Leu, Ile, or Val at position −2, binds only the C-terminal

but not the N-terminal SH2 domain of SHP-1 (*39*), as we have predicted. The excellent agreement between our library results and the literature data clearly demonstrates the validity of our library method.

Two other notable points have been borne out of this work. First, some of the class I peptides selected against the N-terminal SH2 domain (e.g., the pYMXM subset) bear close resemblance to the recognition motifs for the SH2 domains in the p85 subunit of phosphoinositide-3-kinase (*44*). However, upon examination of the pYMXM motifs on PDGF receptor (DGGpY^{740}MDMSK) (*44*−*46*), insulin receptor substrate-1 (NGDpY^{628}MPMSP, PNGpY^{658}MMMSP, TGDpY^{727}-MNMSP, and SEEpY^{939}MNMDL) (*44*, *46*, *47*), and mT antigens (ENEpY^{298}MPMAP from hamster and EEEpY^{315}-MPMED from mouse) (*44*, *45*), which are known to bind to p85 SH2 domains but not to SHP-1, we found that none of these motifs had a leucine or isoleucine at the −2 position. Presumably, these proteins have specifically avoided these hydrophobic residues at position −2 to prevent recruitment of SHP-1, which would prematurely terminate the signaling cascade. It remains to be seen whether the (L/I)XpYMXM motif exists in any proteins, which would be predicted to recruit both SHP-1 and phosphoinositide-3-kinase. Second, our results have convincingly shown that the most frequently selected sequences do not necessarily have the highest binding affinity. For example, selection against the N-terminal SH2 domain resulted in 7 times more class I peptides (total 85) than class II peptides (total 12) and yet, our limited binding studies seem to suggest that the class II peptides are slightly tighter binders than class I peptides (Table 2). Similarly, while out of the 12 class II peptides for the N-terminal SH2 domain, nine contain a leucine and only one has isoleucine at position −2, peptides IYpYANLI and LYpYANLI bind to the SH2 domain with equal affinity.

In summary, we have developed a new combinatorial library methodology for rapid and systematic evaluation of the binding specificity of protein modules such as SH2 domains. This method offers several advantages over the previously reported methods. First, this method gives individual sequences as well as a consensus sequence(s). Second, because selection is based exclusively on affinity, any high-affinity sequence is identified, even if it is present in the library at only minute amounts. This is not true for the methods that employ pool sequencing (*5*, *10*). Previous work has predicted a consensus of pYFXF for the N-terminal SH2 domain of SHP-1, which resembles the class I consensus from this work, but failed to reveal the minor class II sequences (*6*). Finally, our method is rapid and economical. Microsequencing of a large number of beads was not possible in the past due to the prohibitive cost in resources and time associated with the conventional Edman method. By using MALDI-TOF mass spectrometry, however, one can now routinely sequence the peptide from a single resin bead in less than one minute and at a cost of less than five US dollars. This method should be readily applicable to other protein modules and the specificity data obtained will greatly facilitate the identification of the physiological partner proteins of these modular domains.

REFERENCES

1. Bork, P., Schultz, J., and Ponting, C. P. (1997) *Trends Biochem. Sci. 22*, 296−298.
2. Sadowski, I., Stone, J. C., and Pawson, T. (1986) *Mol. Cell. Biol. 6*, 4396−4408.
3. Pawson, T., and Gish, G. D. (1992) *Cell 71*, 359−362.
4. Pawson, T. (1995) *Nature 373*, 573−580.
5. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, L. C. (1993) *Cell 72*, 767−778.
6. Songyang, Z., Shoelson, S. E., McGlade, J., Oliver, P., Pawson, T., Bustelo, X. R., Barbacid, M., Sabe, H., Hanafusa, H., and Yi, T., et al. (1994) *Mol. Cell. Biol. 14*, 2777−2785.
7. Waksman, G., Kominos, D., Robertson, S. C., Pant, N., Baltimore, D., Birge, R. B., Cowburn, D., Hanafusa, H., Mayer, B. J., and Overduin, M., et al. (1992) *Nature 358*, 646−653.
8. Eck, M. J., Shoelson, S. E., and Harrison, S. C. (1993) *Nature 362*, 87−91.
9. Lee, C. H., Kominos, D., Jacques, S., Margolis, B., Schlessinger, J., Shoelson, S. E., and Kuriyan, J. (1994) *Structure 2*, 423−438.
10. Muller, K., Gombert, F. O., Manning, U., Grossmuller, F., Graff, P., Zaegel, H., Zuber, J. F., Freuler, F., Tschopp, C., and Baumann, G. (1996) *J. Biol. Chem. 271*, 16500−16505.
11. Gram, H., Schmitz, R., Zuber, J. F., and Baumann, G. (1997) *Eur. J. Biochem. 246*, 633−637.
12. Kelly, M. A., Liang, H., Sytwu, II, Vlattas, I., Lyons, N. L., Bowen, B. R., and Wennogle, L. P. (1996) *Biochemistry 35*, 11747−11755.
13. Lam, K. S., Salmon, S. E., Hersh, E. M., Hruby, V. J., Kazmierski, W. M., and Knapp, R. J. (1991) *Nature 354*, 82−84.
14. Furka, A., Sebestyen, F., Asgedom, M., and Dibo, G. (1991) *Int. J. Pept. Protein Res. 37*, 487−493.
15. Shen, S. H., Bastien, L., Posner, B. I., and Chretien, P. (1991) *Nature 352*, 736−739.
16. Yi, T. L., Cleveland, J. L., and Ihle, J. N. (1992) *Mol. Cell. Biol. 12*, 836−846.
17. Plutzky, J., Neel, B. G., and Rosenberg, R. D. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1123−1127.
18. Matthews, R. J., Bowne, D. B., Flores, E., and Thomas, M. L. (1992) *Mol. Cell. Biol. 12*, 2396−2405.
19. Beebe, K. D., Shin, J., Peng, J., Chaudhury, C., Khera, J., and Pei, D. (2000) *Biochemistry 39*, 3149−3155.
20. Ottinger, E. A., Shekels, L. L., Bernlohr, D. A., and Barany, G. (1993) *Biochemistry 32*, 4354−4361.
21. Pei, D., Neel, B. G., and Walsh, C. T., (1993) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1092−1096.
22. Pei, D., Lorenz, U., Klingmuller, U., Neel, B. G., and Walsh, C. T. (1994) *Biochemistry 33*, 15483−15493.
23. Pei, D., Wang, J., and Walsh, C. T. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 1141−1145.
24. Hu, Y. J., Wei, Y., Zhou, Y., Rajagopalan, P. T. R., and Pei, D. (1999) *Biochemistry 38*, 643−650.
25. Youngquist, R. S., Fuentes, G. R., Lacey, M. P., and Keough, T. (1995) *J. Am. Chem. Soc. 117*, 3900−3906.
26. Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., and Candia, O. A. (1979) *Anal. Biochem. 100*, 95−97.
27. Malmqvist, M. (1993) *Nature 361*, 186−187.
28. Ladbury, J. E., Lemmon, M. A., Zhou, M., Green, J., Botfield, M. C., and Schlessinger, J. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 3199−2203.
29. Huyer, G., and Ramachandran, C. (1998) *Biochemistry 37*, 2741−2747.
30. Burshtyn, D. N., Yang, W., Yi, T., and Long, E. O. (1997) *J. Biol. Chem. 272*, 13066−13072.
31. Yu, Z., and Chu, Y.-H. (1997) *Bioorg. Med. Chem. Lett. 7*, 95−98.
32. Rajagopalan, P. T., Datta, A., and Pei, D. (1997) *Biochemistry 36*, 13910−13918.
33. Klingmuller, U., Lorenz, U., Cantley, L. C., Neel, B. G., and Lodish, H. F. (1995) *Cell 80*, 729−938.

34. D'Ambrosio, D., Hippen, K. L., Minskoff, S. A., Mellman, I., Pani, G., Siminovitch, K. A., and Cambier, J. C. (1995) *Science 268*, 293−297.
35. Neel, B. G., and Tonks, N. K. (1997) *Curr. Opin. Cell. Biol. 9*, 193−204.
36. Burshtyn, D. N., Lam, A. S., Weston, M., Gupta, N., Warmerdam, P. A. M., and Long, E. C. (1999) *J. Immunol. 162*, 897−902.
37. Hao, B., Gong, W., Rajagopalan, P. T. R., Zhou, Y., Pei, D., and Chan, M. K. (1999) *Biochemistry 38*, 4712−4719.
38. Bone, H., Dechert, U., Jirik, F., Schrader, J. W., and Welham, M. J. (1997) *J. Biol. Chem. 272*, 14470−14476.
39. Mousseau, D., D., Banville, D., LíAbbe, D., Bouchard, P., and Shen, S.-H. (2000) *J. Biol. Chem. 275*, 4467−4474.
40. Taylor, V. C., Buckley, C. D., Douglas, M., Cody, A. J., Simmons, D. L., and Freeman, S. D. (1999) *J. Biol. Chem. 274*, 11505−11512.
41. Law, C. L., Siderenko, S. P., Chandran, K. A., Zhao, Z., Shen, S. H., Fischer, E. H., and Clark, E. A. (1996) *J. Exp. Med. 183*, 547−560.
42. Blasioli, J., Paust, S., and Thomas, M. L. (1999) *J. Biol. Chem. 274*, 2303−2307.
43. Burshtyn, D. N., Scharenberg, A. M., Wagtmann, N., Rajagopalan, S., Berrada, K., Yi, T., Kinet, J. P., and Long, E. O. (1996) *Immunity 4*, 77−85.
44. Piccione, E., Case, R. D., Domchek, S. M., Hu, P., Chaudhuri, M., Backer, J. M., Schlessinger, J., and Shoelson, S. E. (1993) *Biochemistry 32*, 3197−3202.
45. Carpenter, C. L., Auger, K. R., Chaudhuri, M., Yoakim, M., Schaffhausen, B., Shoelson, S., and Cantley, L. C. (1993) *J. Biol. Chem. 268*, 9478−9483.
46. Shoelson, S. E., Sivaraja, M., Williams, K. P., Hu, P., Schlessinger, J., and Weiss, M. A. (1993) *EMBO J. 12*, 795−802.
47. Felder, S., Zhou, M., Hu, P., Urena, J., Ullrich, A., Chaudhuri, M., White, M., Shoelson, S. E., and Schlessinger, J. (1993) *Mol. Cell Biol. 13*, 1449−1455.
48. Blery, M., Kubagawa, H., Chen, C. C., Vely, F., Cooper, M. D., and Vivier, E. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 2446−2451.
49. Philosof-Oppenheimer, R., Hampe, C. S., Schlessinger, K., Fridkin, M., and Pecht, I. (2000) *Eur. J. Biochem. 267*, 703−711.
50. Pumphrey, N. J., Taylor, V., Freeman, S., Douglas, M. R., Bradfield, P. F., Young, S. P., Lord, J. M., Wakelam, M. J., Bird, I. N., Salmon, M., and Buckley, C. D. (1999) *FEBS Lett. 450*, 77−83.
51. Colonna, M., Navarro, F., Bellon, T., Llano, M., Garcia, P., Samaridis, J., Angman, L., Cella, M., and Lopez-Botet, M. (1997) *J. Exp. Med. 186*, 1809−1818.
52. Cella, M., Dohring, C., Samaridis, J., Dessing, M., Brockhaus, M., Lanzavecchia, A., and Colonna, M. (1997) *J. Exp. Med. 185*, 1743−1751.
53. Kuroiwa, A., Yamashita, Y., Inui, M., Yuasa, T., Ono, M., Nagabukuro, A., Matsuda, Y., and Takai, T. (1998) *J. Biol. Chem. 273*, 1070−1074.
54. Xu, M.-J., Zhao, R., and Zhao, Z., (2000) *J. Biol. Chem. 275*, 17440−17446.
55. Veillette, A., Thibaudeau, E., and Latour, S. (1998) *J. Biol. Chem. 273*, 22719−22728.
56. Huber, M., Izzi, L., Grondin, P., Houde, C., Kunath, T., Veillette, A., and Beauchemin, N. (1999) *J. Biol. Chem. 274*, 335−344.

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