Decoding Protein−Protein Interactions through Combinatorial Chemistry: Sequence Specificity of SHP-1, SHP-2, and SHIP SH2 Domains†

Michael C. Sweeney,[‡,§] Anne-Sophie Wavreille,[‡] Junguk Park,[‡] Jonathan P. Butchar,[||] Susheela Tridandapani,[§,||] and Dehua Pei*[,‡,§]

Departments of Chemistry and Internal Medicine and Ohio State Biochemistry Program, The Ohio State University, 100 West 18th Avenue, Columbus, Ohio 43210

Received July 19, 2005; Revised Manuscript Received September 2, 2005

ABSTRACT: A general, combinatorial library method for the rapid identification of high-affinity peptide ligands of protein modular domains is reported. The validity of this method has been demonstrated by determining the sequence specificity of four Src homology 2 (SH2) domains derived from protein tyrosine phosphatase SHP-1 and SHP-2 and inositol phosphatase SHIP. A phosphotyrosyl (pY) peptide library was screened against the SH2 domains, and the beads that carry high-affinity ligands of the SH2 domains were identified and peptides were sequenced by partial Edman degradation and mass spectrometry. The results reveal that the N-terminal SH2 domain of SHP-2 is capable of recognizing four different classes of pY peptides. Binding competition studies suggest that the four classes of pY peptides all bind to the same site on the SH2 domain surface. The C-terminal SH2 domains of SHP-1 and SHP-2 and the SHIP SH2 domain each bind to pY peptides of a single consensus sequence. Database searches using the consensus sequences identified most of the known as well as many potential interacting proteins of SHP-1 and/or SHP-2. Several proteins are found to bind to the SH2 domains of SHP-1 and SHP-2 through a new, nonclassical ITIM motif, (V/I/L)XpY(M/L/F)XP, which corresponds to the class IV peptides selected from the pY library. The combinatorial library method should be generally applicable to other protein domains.

Protein−protein interactions are an integral component of many cellular processes such as intracellular signaling. Frequently, the interactions are mediated by modular domains, which recognize small, specific peptide motifs in their partner proteins. The Src homology 2 (SH2) domain was one of the first examples of such domains, which binds to specific phosphotyrosyl (pY)[1] peptides (*1*). A large number of SH2 domains are now known, and it has been estimated that the human genome encodes at least 115 SH2 domains (*2*). Each SH2 domain interacts with a unique subset of pY peptides, and the sequence specificity is primarily determined by the three amino acids immediately C-terminal to pY. Since the initial discovery of the SH2 domain, some 30 other types of modular domains have now been discovered (e.g., SH3, PDZ, FHA, and PTB domains), many of which also recognize various peptide motifs in their target proteins (*3*). However, for the vast majority of these domains, their sequence specificity or in vivo interaction partners are currently unknown.

One approach to sorting out the complex protein−protein interaction network is to determine the sequence specificity of these modular domains through the screening of combinatorial peptide libraries and then use the consensus sequence(s) to search the protein databases. Several combinatorial methods have been reported. In their pioneering work with SH2 domains, Cantley and co-workers employed affinity columns containing an immobilized SH2 domain to enrich SH2-binding sequences from a pY peptide library (*4*), a technique later expanded upon by others (*5*). Sequencing of the enriched peptide pool by conventional Edman degradation reveals the preferentially selected amino acid(s) at each position. A variation of this method involved screening support-bound libraries against a fluorescently labeled SH2 domain (*6*). The positive beads with the bound SH2 were removed from the library using a fluorescence-activated bead sorter, and all of the selected beads were pooled and sequenced by Edman degradation. This method of sequencing provides information on the most preferred amino acid(s) at each position but, importantly, does not give individual sequences. Since the method selects for *both* affinity and abundance of certain types of sequences, a high-affinity peptide of low abundance may not emerge from the consensus sequence(s). A second method involves the iterative synthesis and screening of sublibraries or "positional scanning" (*7*). However, this method suffers from the same

† This work was supported by National Institutes of Health grants R01 GM062820, R01 AI059406, T32 GM08512 (M. C. S.), and RR15895.

* To whom correspondence should be addressed at the Department of Chemistry, The Ohio State University. Telephone: (614) 688-4068. Fax: (614) 292-1532. E-mail: pei.3@osu.edu.

‡ Department of Chemistry.

§ Ohio State Biochemistry Program.

|| Department of Internal Medicine.

[1] Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; Nic-OSU, *N*-hydroxysuccinimidyl nicotinate; MBP, maltose-binding protein; IPTG, isopropyl *β*-D-thiogalactoside; ITIM, immunoreceptor tyrosine-based inhibition motif; pY, phosphotyrosine; PITC, phenyl isothiocyanate; SH2 domain, Src homology 2 domain; SPR, surface plasmon resonance; TFA, trifluoroacetic acid.

10.1021/bi051408h CCC: $30.25
Published on Web 10/21/2005

drawbacks as the first method, in addition to being highly labor intensive. In a third method, bacteriophage bearing short random peptide sequences on their surfaces were selected against an immobilized modular domain (*8*−*10*). The sequences of the binding peptides were determined by amplifying the bound phage and sequencing their DNA. This method is highly effective for modular domains that recognize unmodified peptides but generally does not work with protein domains that recognize posttranslationally modified peptides (*10*, *11*). Here we describe another method, in which resin-bound peptide libraries are selected against a protein receptor and the positive beads are removed from the library and sequenced by partial Edman degradation, a high-throughput technique recently developed by one of these laboratories. Our method produces a large number of individual sequences, from which a consensus sequence(s) can be derived. This method is applied to determine the sequence specificity of four SH2 domains from phosphatases SHP-1, SHP-2, and SHIP.

EXPERIMENTAL PROCEDURES

Materials. The pMAL-c2 vector, all DNA modifying enzymes, and amylose resin were purchased from New England Biolabs. The pET-28a vector and *Escherichia coli* BL21(DE3) Rosetta CodonPlus strain were purchased from Novagen. All oligonucleotides were purchased from Integrate DNA Technologies. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP), antibiotics, *N*-hydroxysuccinimido-biotin, Sephadex G-25 resin, 4-hydroxy-α-cyanocinnamic acid, and organic solvents were obtained from Sigma-Aldrich. Talon resin for IMAC purification was purchased from Clontech. Reagents for peptide synthesis were from Advanced ChemTech, Peptides International, and NovaBiochem. *N*-Hydroxysuccinimidyl nicotinate (Nic-OSU) was from Advanced ChemTech and was recrystallized from ethyl acetate prior to use. Phenyl isothiocyanate was purchased in 1 mL sealed ampules from Sigma-Aldrich and used without purification. Protein concentration was determined by the Bradford method using bovine serum albumin (Sigma-Aldrich) as standard. The Raw 264.7 murine macrophage cell line was obtained from ATCC and maintained in RPMI supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin, as described previously (*12*).

SH2 Domain Constructs. A pET-MAL vector was created by subcloning the *malE* gene from pMAL-c2 into pET-28a with the retention of all pMAL-c2 multiple cloning sites. The DNA sequences coding for the SHP-2 N-SH2 domain (aa 1−106), C-SH2 domain (aa 108−220), and SHIP SH2 domain (aa 1−109) were isolated by polymerase chain reaction (PCR) from plasmids pET22-SHP2 (*13*) and pGEX2T-SHIP (*14*), respectively. The DNA primers used were as follows: N-SH2, T7 promoter primer, and 5′-AGATTAGAAGCTTTCAATCTGCACAGTTCAGAGGATATTTAAGCA-3′; C-SH2, 5′-ATATAGAATTCATGACCTCTGAAAGGTGGTTTCATGGACA-3′ and 5′-AGATTAGAAGCTTTCAACGAGTCGTGTTAAGGGGCTGCT-3′; SHIP SH2, 5′-GCGAATTCATGCCTGCCATGGTCCCTGG-3′ and 5′-CGTCCAAGCTTCACTCCTCCTCCAGGGGCAC-5′. The PCR products were digested with restriction endonucleases *Eco*RI (*Nde*I in the case of N-SH2 domain) and *Hin*dIII and ligated into their corresponding sites in pET-MAL. This procedure resulted in the fusion of the SH2 domains to the C-terminus of maltose-binding protein (MBP), facilitating both purification and biotinylation. In addition, each SH2 domain was constructed in its isolated form containing an N-terminal six-histidine tag. This was carried out in a similar manner as described above, except that the PCR products were ligated into plasmid pET-28a instead. The identity of all DNA constructs was confirmed by dideoxy sequencing. SHP-1 SH2 domain constructs have previously been described (*15*). Recombinant full-length SHP-1 and SHP-2 were prepared as previously described (*13*, *15*).

Purification and Biotinylation of MBP-SH2 Proteins. E. coli BL21(DE3) cells harboring the proper pET-MAL-SH2 plasmid were grown in LB medium to the mid-log phase and induced by the addition of 300 μM isopropyl β-D-thiogalactoside (IPTG) for 2.5 h at 30 °C. The cells were harvested by centrifugation and lysed in the presence of protease inhibitors by passing through a French press. The MBP-SH2 protein was purified from the crude lysate on an amylose column according to manufacturer's recommended procedures. The protein was concentrated in an Amicon concentrator to approximately 4 mg/mL (in 20 mM HEPES, pH 8.2, 150 mM NaCl, 2 mM 2-mercaptoethanol, and 10 mM maltose) and treated with 2 equiv of *N*-hydroxysuccinimido-biotin at room temperature for 45 min. Excess biotin was removed by passing the solution through a Sephadex G-25 column. After concentration and addition of glycerol (final 40%), the protein was quickly frozen in a dry ice/2-propanol bath. MBP alone was prepared and labeled in the same manner as a control.

Purification of Histidine-Tagged SH2 Domains. N-Terminally histidine-tagged SH2 domains were expressed in a Rosetta CodonPlus strain of *E. coli* BL21(DE3) cells. Protein expression was induced by the addition of 300 μM IPTG at 30 °C for 3 h. The cells were lysed in a French pressure cell, and the crude lysate was loaded onto a Talon cobalt affinity column (10 mL). After extensive washing, the SH2 protein was eluted with 125 mM imidazole and passed through a size exclusion column (XK-16 Superdex-75) connected to an FPLC system (Pharmacia). The elution buffer contained 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 10 μM tris(carboxyethyl)phosphine. All proteins were flash frozen without the addition of glycerol.

Synthesis of the pY Library. The library was synthesized on 5 g of 90 μm TentaGel S NH_2 resin using standard Fmoc chemistry employing HBTU/HOBt/DIPEA as the coupling reagents. The invariant positions (LNBBRM and pY) were synthesized with 4 equiv of Fmoc-amino acids, and the coupling reaction was terminated after ninhydrin tests were negative. The random positions were synthesized using the split-synthesis method (*7*, *16*, *17*). The coupling reactions employed 5 equiv of Fmoc-amino acids and were allowed to proceed for 45 min, after which the coupling reaction was repeated once to ensure complete reaction. To facilitate sequence determination by mass spectrometry, 5% Ac-Gly was added to the coupling reactions of Leu and Lys, whereas 5% Ac-Ala was added to the coupling reactions of Nle (*18*). After removal of the terminal Fmoc group, the resin-bound library was washed with dichloromethane and deprotected using reagent K [7.5% phenol, 5% water, 5% thioanisole, 2.5% ethanedithiol, 1% anisole, and 1% triisopropylsilane in trifluoroacetic acid (TFA)] at room temperature for 60 min. The library was washed with trifluoroacetic acid,

dichloromethane, and methanol before drying for storage at −20 °C.

Library Screening. In a micro-BioSpin column (0.8 mL, Bio-Rad), 100 mg of the pY library was swollen in dichloromethane, washed extensively with methanol, ddH_2O, and HBST buffer (30 mM HEPES, pH 7.4, 150 mM NaCl, and 0.05% Tween 20), and blocked for 1 h with 800 μL of HBST buffer containing 0.1% gelatin. The resin was drained and resuspended in 800 μL of a biotinylated MBP-SH2 domain of interest (10−50 nM final concentration) in HBST buffer plus 0.1% gelatin. After overnight incubation at 4 °C with gentle mixing, the resin was drained and resuspended in 800 μL of SAAP buffer (30 mM Tris, pH 7.6, 1 M NaCl, 10 mM $MgCl_2$, 70 μM $ZnCl_2$, and 20 mM potassium phosphate) containing 1 μL of streptavidin−alkaline phosphatase (Prozyme, ∼1 mg/mL). After 10 min of gentle mixing at 4 °C, the resin was rapidly drained and washed with 400 μL of SAAP buffer, 400 μL of HBST buffer, and 400 μL of staining buffer (30 mM Tris, pH 8.5, 100 mM NaCl, 5 mM $MgCl_2$, and 20 μM $ZnCl_2$). The resin was then transferred into a 35 mm Petri dish by rinsing with 5 × 300 μL of the staining buffer. Upon the addition of 80 μL of 5 mg/mL BCIP in the staining buffer, intense turquoise color developed on positive beads in ∼45 min, when the staining reaction was quenched by the addition of 3 mL of 8 M guanidine hydrochloride. The resin was transferred back into the BioSpin column, extensively washed with water, and replated in the Petri dish, from which colored beads were picked manually using a pipet under a dissecting microscope. The positive beads were sorted by color intensity into "intense", "medium", and "light" categories. Control experiments with biotinylated MBP produced no colored beads under identical conditions.

Partial Edman Degradation and Peptide Sequencing. The positive beads from each color intensity category were pooled and subjected to partial Edman degradation as previously described (*18*). The beads were suspended in 66% pyridine (aq) containing 0.1% Et_3N, to which was added an equal volume of 5% phenyl isothiocyanate (PITC) in pyridine containing a variable amount of Nic-OSU. After rapid mixing, the reaction was allowed to proceed for 6 min. The beads were washed with methanol, dichloromethane, and TFA and suspended in TFA (2 × 6 min). After extensive washing with CH_2Cl_2 and pyridine, the cycle was repeated. An optimized procedure was established for this library by trial and error using unselected beads and employed varying PITC/Nic-OSU ratios as follows: 6:1 for the N-terminal T and A; 4.5:1 for the N-terminal random positions; no Nic-OSU during pY degradation; and 5:1 for the C-terminal random positions. Finally, the linker sequence was capped by Nic-OSU in the absence of PITC. The beads were then treated for 20 min with ∼1 mL of TFA containing NH_4I (10 mg) and Me_2S (20 μL) on ice to reduce any oxidized methionine. The beads were washed with ddH_2O, placed in individual microcentrifuge tubes, and treated overnight in the dark with 20 μL of 70% TFA containing CNBr (20 mg/mL). After evaporation to dryness, the peptides were dissolved in 5 μL of 0.1% TFA in water. One microliter of the peptide solution was mixed with 2 μL of 0.1% TFA in 50% acetonitrile saturated with 4-hydroxy-α-cyanocinnamic acid and spotted onto a 96-well sample plate. Mass spectrometry was performed on a Bruker Reflex III MALDI-TOF instrument in an automated manner. Sequence determination from the mass spectra was performed manually.

Synthesis of Biotinylated pY Peptides. All pY peptides contained a common C-terminal linker, -LNBKR-NH_2. Each peptide was synthesized on ∼65 mg of CLEAR-amide resin using standard Fmoc/HBTU/HOBt chemistry. The N-terminus was acetylated by the treatment of Ac_2O. Cleavage and deprotection were carried out using reagent K as described above. Approximately 3 mg of the crude peptide was dissolved in a minimal volume of DMSO (300−500 μL, with sonication) and reacted with 1 equiv of NHS-PEG_4-biotin (Quanta Biochem) in 25 μL of DMSO. After 45 min at room temperature, the mixture was triturated twice with 20 volumes of Et_2O. The precipitate was collected and dried under vacuum. The biotinylated pY peptide was purified by reversed-phase HPLC on a C_{18} column (Vydac 300 Å, 4.6 × 250 mm). The identity of each peptide was confirmed by MALDI-TOF mass spectrometric analysis. This procedure resulted in the addition of a 15-atom hydrophilic linker between the side chain of the C-terminal lysine and the carboxyl group of biotin.

Determination of Dissociation Constants by Surface Plasmon Resonance (SPR). All measurements were made with the isolated SH2 domains containing an N-terminal histidine tag at room temperature on a BIAcore 3000 instrument. A sensorchip containing immobilized streptavidin was conditioned with 1 M NaCl in 50 mM NaOH (aq) according to manufacturer's instructions. The biotinylated pY peptides were immobilized onto the sensorchip by flowing 6 μL of ∼8 μM pY peptide solution in HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% polysorbate 20). Data for the secondary plot analysis were acquired by passing increasing concentrations (0−5 μM) of an SH2 protein in HBS-EP buffer over the sensorchip for 2 min at a flow rate of 15 μL/min. A blank flow cell (no immobilized pY peptide) was used as control to correct for any signal due to the solvent bulk and/or nonspecific binding interactions. In fact, neither significant bulk effect nor nonspecific binding was observed. In between two runs, the sensorchip surface was regenerated by flowing a strip solution (10 mM NaCl, 2 mM NaOH, and 0.025% SDS in H_2O) for 5−10 s at a flow rate of 100 μL/min. The equilibrium response unit (RU_{eq}) at a given SH2 protein concentration was obtained by subtracting the response of the blank flow cell from that of the sample flow cell. The dissociation constant (K_D) was obtained by nonlinear regression fitting of the data to the equation:

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

where RU_{eq} is the measured response unit at a certain SH2 protein concentration and RU_{max} is the maximum response unit.

Peptide Pull-Down Assays. Briefly, Raw 264.7 cells were lysed in TN1 lysis buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 10 mM Na_3VO_4, and 10 μg/mL each of aprotinin and leupeptin). Nuclei were removed by centrifugation for 10 min at 13000 rpm at 4 °C. Equal amounts of protein from each sample were incubated overnight with 5 μg of biotinylated pY peptides at 4 °C. Streptavidin−agarose beads were added to the samples, which were then incubated

for 1 h at 4 °C. Beads were washed twice in TN1 buffer and boiled in SDS−PAGE sample-loading buffer (60 mM Tris, pH 6.8, 2.3% SDS, 10% glycerol, 0.01% bromophenol blue, and 1% 2-mercaptoethanol) for 5 min, and the eluted proteins were separated by SDS−PAGE. Following gel electrophoresis, proteins were transferred to nitrocellulose membranes and incubated overnight at 4 °C with anti-SHP1 (rabbit polyclonal antibody from Upstate Biotechnology), anti-SHP2 (rabbit polyclonal antibody from Santa Cruz Biotechnology), or anti-SHIP (rabbit polyclonal antibody, a kind gift from Dr. K. Mark Coggeshall, Oklahoma Medical Research Foundation, Oklahoma City, OK). After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies, washed again, and briefly incubated in ECL (Amersham) for chemiluminescent detection on X-ray films. For experiments involving purified SHP-1 and SHP-2 proteins, 5 μg of biotinylated pY peptide was incubated with 1 μg of the purified proteins in TN1 buffer. The pY peptide−protein complexes were captured with streptavidin−agarose beads and analyzed as described above.

RESULTS

Library Design, Synthesis, and Screening. To demonstrate the effectiveness of the combinatorial method, we chose to determine the sequence specificity for the SH2 domains of protein tyrosine phosphatases SHP-1 and SHP-2 and inositol phosphatase SHIP. SHP-1 and SHP-2 belong to a subfamily of PTPs which each contain two SH2 domains N-terminal to their catalytic domain, whereas SHIP contains a single SH2 domain. All three proteins are involved in a variety of signaling pathways (*19*). Despite their sequence homology, SHP-1 and SHP-2 have very different in vivo functions. For example, SHP-2 generally acts as a *positive* regulator for the various signaling pathways, whereas SHP-1 primarily acts as a *negative* regulator of signaling events (*19*). Some studies show that SHP-1, SHP-2, and SHIP recognize distinct pY motifs on various receptors via their SH2 domains, while others report that the three enzymes can compete for binding to a common receptor bearing one or more immunoreceptor tyrosine-based inhibition motifs (ITIMs) (*20*). These data suggest that the SH2 domains in SHP-1, SHP-2, and SHIP have distinctive but partially overlapping specificities. Therefore, a detailed study on their sequence specificities would be very helpful in identifying their physiological targets and determining their cellular functions.

The specificity of an SH2 domain is primarily determined by the pY residue and the three residues immediately C-terminal to pY (*21*−*23*), although it has been reported that, for a few SH2 domains including those of SHP-1 and SHP-2, the −2 position (two residues N-terminal to pY, which is position 0) is also important for high-affinity interaction (*15*, *24*). Thus, we designed a pY library, H_2N-TAXXpYXXX-LNBBRM-resin, where X represents norleucine (Nle) or any of the 18 natural amino acids except for Met and Cys and B is β-alanine. The N-terminal dipeptide (TA) helps to reduce potential bias caused by electrostatic interactions between an SH2 protein and the free N-terminus (which is required for peptide sequencing). At the C-terminus, a methionine permits the release of peptides from the resin by CNBr treatment prior to sequencing, while arginine serves to increase peptide solubility and sensitivity during MALDI-MS sequencing by providing a fixed positive charge. The two β-alanines add flexibility to the peptides, making them more accessible to a protein target. The dipeptide LN is added to shift the masses of the peptides to >600 Da, so that their mass spectral peaks do not overlap with matrix signals (vide infra). Methionine is excluded from the randomized positions to avoid internal cleavage during CNBr treatment and is replaced by the isosteric Nle residue. The library was synthesized on TentaGel S NH_2 resin (~90 μm in diameter and ~2.86×10^6 beads/g) using the split-pool method (*7*, *16*, *17*) with each bead carrying ~100 pmol of a unique sequence. This method ensures equal representation of all possible sequences in the library.

The theoretical diversity of the above library is 19^5 or 2.5×10^6. A typical screening involved ~100 mg of resin, which covers ~11% of the sequence space. The resin was incubated with a small amount of an SH2 domain protein (10−50 nM final concentration), constructed as an MBP fusion protein, and biotinylated on a surface lysine residue(s). Binding of the biotinylated SH2 domain to a resin-bound pY peptide recruits a streptavidin−alkaline phosphatase conjugate to the surface of that bead. Upon the addition of BCIP, the bound alkaline phosphatase cleaves BCIP into an indole, which dimerizes to form a turquoise precipitate deposited on the bead surface. As a result of this reaction cascade, beads that carry high-affinity SH2 ligands become colored. The number of colored beads depends on the binding affinity and specificity of the protein domain as well as the stringency of the screening conditions (e.g., SH2 domain concentration, number of washings, and length of staining time). The screening reactions were controlled so that 10−100 colored beads were obtained from 100 mg of resin (~286000 beads). The number of positive beads was quite reproducible when multiple screenings were performed against the same SH2 domain. Positive beads were manually removed from the library using a micropipet with the aid of a dissecting microscope.

Peptide Sequencing by Partial Edman Degradation and Mass Spectrometry. Individual sequence determination for a large number of selected beads presented an insurmountable challenge in the past, because Edman sequencing is both expensive and time-consuming. To this end, we have recently developed an inexpensive, high-throughput peptide sequencing technique, termed "partial Edman degradation", which is ideally suited for sequencing resin-bound peptides (*18*). Briefly, resin-bound peptides are treated with a ~5:1 mixture of PITC and Nic-OSU followed by TFA (Figure 1a). This results in the removal of the N-terminal amino acid from 90% to 95% of the peptides (Edman degradation); for the remaining 5−10% of the peptides on each bead, reaction with Nic-OSU results in permanent N-terminal acylation and retention of the N-terminal residue (no degradation). Repetition of the above procedure produced a peptide ladder, which was subsequently analyzed by MALDI mass spectrometry. Figure 1b shows the mass spectrum of a single bead carrying the sequence TA(Nle)YpYATILNBBRM. Note that the isobaric residues Nle, Leu, and Ile are unambiguously resolved in the spectra by their appearance as a singlet (Ile) vs doublet peaks (Nle and Leu) (*18*).

Specificity of the C-SH2 Domain of SHP-2. Screening of the above library (100 mg) against 10 nM C-terminal SH2 domain of SHP-2, MBP-CSH2, resulted in 14 intensely colored beads, 12 lightly colored beads, and 53 beads of

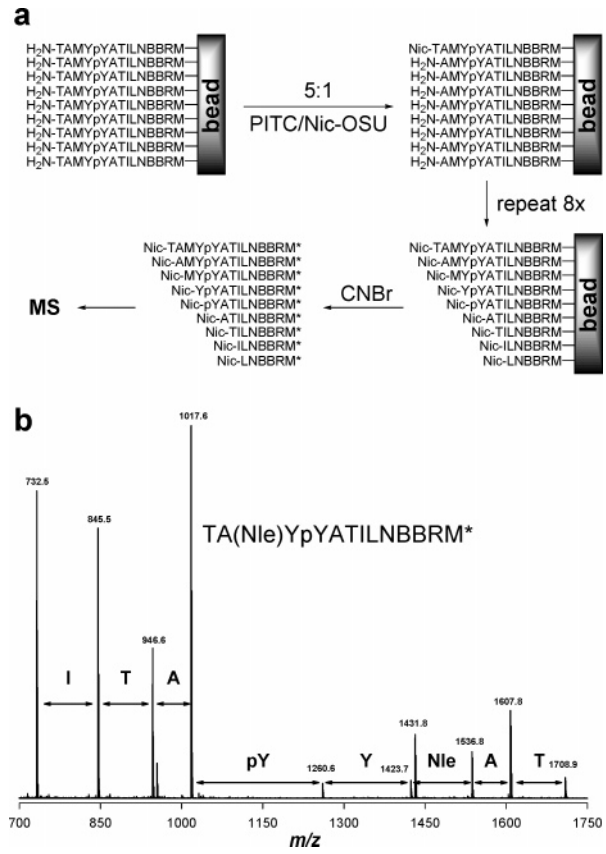

FIGURE 1: Peptide sequencing by partial Edman degradation and mass spectrometry. (a) Scheme showing the reactions involved in the degradation of a resin-bound peptide, TAMYpYATILNBBRM. (b) MALDI mass spectrum of the peptide and its truncation products (derived from a single 90 μm bead). A doublet at *m*/*z* 1423.7 and 1431.8 indicates that the residue N-terminal to tyrosine is a norleucine. Key: M, methionine at the C-terminus or norleucine at internal positions; M*, homoserine lactone.

intermediate color intensity. The beads (79 total) were separated into three pools according to their color intensity ("intense", "intermediate", and "light"), placed in three different vessels, and subjected to partial Edman degradation. The degraded beads were then separated into individual microcentrifuge tubes and treated with CNBr, and the released peptides were analyzed by MALDI-TOF MS. Out of the 79 samples, 77 produced high-quality spectra, allowing for unambiguous determination of their peptide sequences (Table 1). The mass spectra for the remaining two beads had one or more peaks missing, preventing complete sequence assignment. The 77 sequences were sorted according to the frequency of amino acid occurrence at position +3 followed by alphabetical order at positions +1 and +2 using the Microsoft Excel program. Inspection of selected sequences indicates that this SH2 domain strongly prefers a nonpolar aliphatic residue at the +3 position, with isoleucine being the most preferred amino acid (present in 57 selected peptides), followed by valine (present in 15 peptides) and leucine (present in 5 sequences) (Figure 2). The +1 position has the second most stringent requirement, strongly preferring an alanine (present in 46 peptides) or other small amino acids such as serine (present in 18 sequences), threonine (present in 10 sequences), and valine (present in 3 sequences). The −2 position is also critical for binding to the C-SH2 domain of SHP-2, preferring a β-branched amino acid such as threonine, valine, and isoleucine, which are occasionally replaced by a tyrosine. There is a weak preference for a β-branched residue at the +2 position and virtually no selectivity at the −1 position.

Table 1: Selected SHP-2 C-SH2 Domain-Binding Sequences (77 Total)[a]

VIpYANI	**VLpYAII**	VTpYSQI	TRpYAVV
VIpYANI	VYpYAII	*YTpYSQI*	TYpYAVV
IEpYAQI	VYpYAII	*VEpYSEI*	VTpYAIV
YTpYAQI	**TQpYAII**	TYpYSMI	*IHpYATV*
AIpYASI	SNpYAII	TFpYSRI	*TVpYASV*
YSpYASI	MYpYAII	YYpYSRI	*TRpYAKV*
IWpYASI	YQpYAII	TRpYTQI	IIpYSQV
THpYASI	INpYAMI	VIpYTQI	VIpYSSV
TIpYATI*	THpYAMI	VTpYTSI	VIpYSVV
TSpYATI	TMpYAMI	VFpYTTI	TQpYSIV
VTpYATI	TTpYAAI	HFpYTTI	TVpYSIV
VGpYATI	*YKpYARI*	TIpYTVI	TIpYSMV
LYpYATI	*YMpYAHI*	TIpYTII	TVpYSEV
*NApYATI**	YMpYAEI	TYpYTMI	TVpYTEV
VApYAVI	ILpYSTI	TIpYTEI	TVpYASL
VHpYAVI	TTpYSTI	TYpYVEI	VYpYATL
IApYAVI	VHpYSTI	IQpYVQI	YLpYATL
IHpYAVI	TYpYSSI	TKpYVVI	IQpYAVL
PIpYAVI	**IVpYSQI**	TLpYAVV	TApYAIL
NMpYAVI			

[a] All sequences were obtained from a screening experiment performed with 10 nM SHP-2 C-SH2 domain. Key: bold type, peptides from the most intensely colored beads; roman type, peptides from beads of medium color intensity; italic type, sequences from the lightly colored beads; *, peptides selected for SPR analysis; M, norleucine.

To test whether the library screening result is reproducible, the above experiment was repeated with 50 nM MBP-CSH2 protein under otherwise identical conditions. Ninety intensely colored and ~150 less colored beads were obtained and sequenced (see Table S1 in Supporting Information for individual sequences). A plot of the frequency of appearance for each amino acid (based on the 90 intensely colored beads) produced a pattern indistinguishable from that derived from the 10 nM screening (Figure 2). These results allow us to draw the following conclusions. First, the SHP-2 C-SH2 domain recognizes a single consensus sequence (T/V/I/y)-XpY(A/s/t/v)X(I/v/l), where lower case letters represent less frequently selected residues and X is any amino acid except for glycine and proline. Second, the screening method is highly reproducible and robust. Finally, one can unambiguously determine the sequence specificity of an SH2 domain by screening just a fraction of the complete library (~11% in this case), because not all of the randomized positions are crucial for SH2 binding. The same conclusion (the validity of using incomplete libraries) was also borne out of our earlier work with FHA domains (*25*). This greatly reduces the cost and time required for the characterization of each SH2 domain.

Specificity of the N-SH2 Domain of SHP-2. Initial screening of 100 mg of the library against 10 nM SHP-2 N-SH2 domain gave rather surprising results; the N-SH2 domain appeared to bind pY peptides of several distinct classes. To obtain additional sequences for more reliable analysis, the screening experiment was repeated twice, once at 10 nM and another at 50 nM N-SH2 protein. Again, the results were highly reproducible, with all three screenings producing the same types of sequences. All together, 150 intensely colored beads were selected from 300 mg of the library, and their

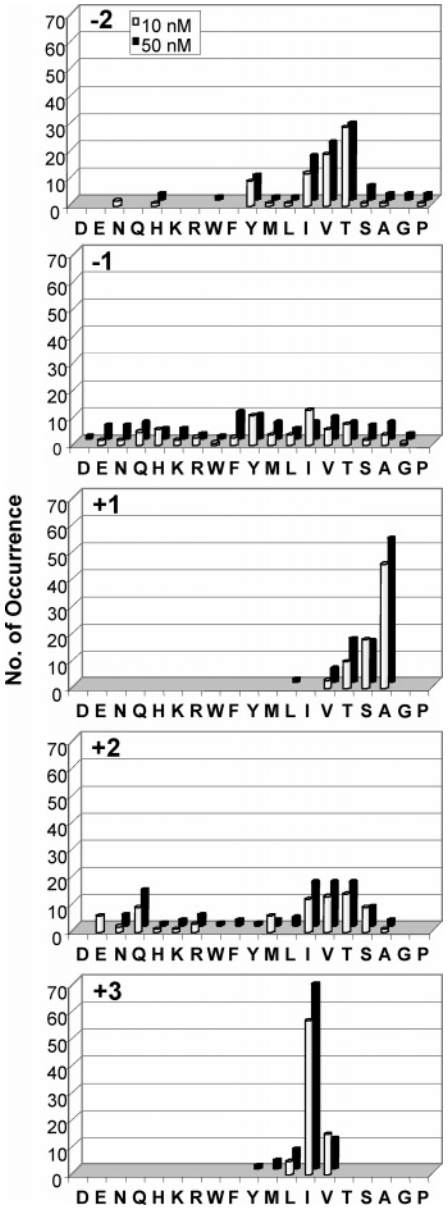

FIGURE 2: Specificity of the C-SH2 domain of SHP-2. Displayed are the amino acids identified at each position from −2 to +3 relative to pY (position 0). Number of occurrence on the *y* axis represents the number of selected sequences that contain a particular amino acid at a certain position. Key: open bar, results from screening at 10 nM C-SH2 protein (total 77 sequences); closed bar, results from screening at 50 nM C-SH2 protein (total 90 sequences); M, norleucine.

sequences are listed in Table 2 (the most colored beads from 10 nM screenings are shown in boldface). Additional sequences from less colored beads are listed in Table S2 under Supporting Information. The selected sequences can be sorted into four different classes using the Excel program by grouping homologous sequences together. The most abundant class (class I) has a consensus sequence of (I/L/V/m)XpY(T/V/A)X(I/V/L/f), which is similar to that of the C-SH2 domain, albeit with some subtle differences at the −2 and +1 positions (Figure 3). While the C-SH2 domain most prefers a threonine at the −2 position, threonine was not found among any of the N-SH2-binding peptides (class I). On the other hand, leucine is seldom selected at the −2 position by the C-SH2 domain but is one of the two most

Table 2: Selected SHP-2 N-SH2 Domain-Binding Sequences (150 Total)[a]

class I		class II	class III	class IV
IVpYADI	**LRpYIQV**	**WIpYFIR**	ITpYLIG	LVpYMGP
LFpYAEI	IFpYTAV	VQpYFIR	YTpYLVA	LHpYMGP
VMpYAEI	ILpYTEV	WMpYKIY	**IHpYLYA***	ALpYMIP
LHpYAII	ITpYTEV	**WSpYKIY**	**TLpYLYA**	**ILpYMIP**
VVpYAII	LVpYTEV*	WMpYNIG	LNpYLYM	WMpYMMP
VTpYALI	IYpYTPV	WTpYQIL*	**VLpYLYP**	VLpYMQP*
LYpYANI	YIpYTTV	WTpYQIT	**IFpYLYS**	VMpYMQP
IRpYAQI	IRpYTYV	WVpYRID	VMpYLYS	**TEpYMVP**
MYpYARI	MNpYVIV	**WMpYRII***	IVpYLYT	**ILpYFIP**
SYpYASI	WSpYVLV	WIpYTIG	PMpYMIA	IVpYFVP
LVpYATI*	MHpYVQV	**WVpYTIN**	VVpYMYS	**VIpYFVP***
LYpYATI	LRpYVRV	WTpYVIT	VTpYMYT	IIpYFYP
MYpYATI	LHpYVSV	WVpYYIG	**VVpYMYT**	
MYpYATI	LRpYVSV	WMpYYIQ	IIpYTIG	
YApYATI	WMpYYQV	WVpYYIR	IKpYTYP	
LNpYAVI	LRpYAKL	WMpYQLS	IMpYTYP	
LRpYAVI	IVpYAML	WVpYRLE	ITpYTYP*	
ISpYIEI	VIpYAQL	WMpYRLI	YVpYTYT	
LNpYIVI	LRpYMQL	ITpYRLV		
LYpYLQI	IQpYMVL	WMpYRLY		
LNpYMTI	IVpYTLL	**WTpYSLA**		
IFpYTAI	VNpYTTL	WTpYSLQ		
YVpYTAI	IApYVEL	WTpYSLY		
IMpYTDI	**IRpYVEL**	WMpYTLN		
IYpYTDI	**VApYVEL***	WTpYVLY		
VYpYTEI	IQpYVML	WTpYYLF		
IMpYTII	IQpYVML	WTpYYLI		
VTpYTII	INpYVQL	WMpYYLT		
VTpYTLI	VTpYVQL	WMpYYLY		
VYpYTQI	MNpYVTL	WMpYRMN		
ISpYTYI	RApYIVM	WTpYVTS		
ITpYTYI	LYpYATF	WIpYYTR		
INpYVEI	LNpYMTF*	WVpYYTY		
IHpYVMI	MSpYMVF	WTpYQYV		
INpYVQI	YNpYMVF	WMpYRYQ		
IWpYVSI	LYpYTSF	WTpYSYT		
LRpYVSI	LNpYVIF			
LTpYVTI	LNpYVLF			
IIpYVVI	ITpYLVY			
LYpYAQV	LRpYLVY			
INpYIEV	**QMpYYLY**			
ISpYIEV	LYpYYQY			

[a] Boldfaced sequences represent the intensely colored beads from screenings under the most stringent conditions (10 nM N-SH2 domain). Peptides labeled with asterisks were selected for SPR analysis. M = norleucine.

preferred residues for the N-SH2 domain. At the +1 position, while the C-SH2 domain strongly prefers alanine to serine, threonine, or valine, the N-SH2 domain selected threonine, valine, and alanine with equal frequency (but not serine). Sequence covariance is also observed, with pY(A/T)XI, pY(T/V)XV, pYVXL, and pY(M/V)XF motifs being frequently selected.

The second most abundant class of peptides (class II) has the consensus of W(M/T/v)pY(y/r)(I/L)X, where the −2 residue is almost always a tryptophan and the −1 position is usually norleucine, threonine, or valine (Table 2 and Figure 3). Remarkably, while the +2 position is highly variable among class I peptides, it is the most invariant position on the C-terminal side of pY for class II peptides. The identity of the most preferred residues (Ile and Leu) suggests that the +2 side chain is engaged in hydrophobic interactions with the SH2 domain. Consistent with this binding mode, the selected +1 and +3 residues are variable and contain predominantly hydrophilic (e.g., Tyr, Arg, Gln, and Thr) or small side chains, suggesting that they presumably face the

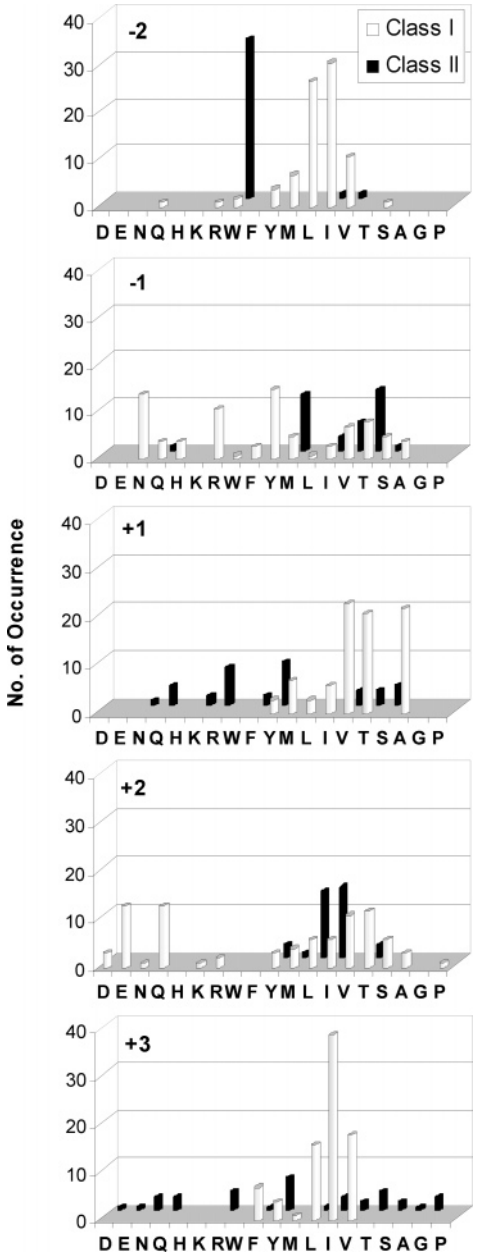

FIGURE 3: Specificity of the N-SH2 domain of SHP-2. Displayed are the amino acids identified at each position (−2 to +3). Number of occurrence on the *y* axis represents the number of selected sequences that contain a particular amino acid at a certain position. Key: open bar, class I peptides; closed bar, class II peptides; M, norleucine.

solvent. Class III peptides have the consensus sequence of (I/V)XpY(L/M/T)Y(A/P/T/S/g) (Table 2). A distinctive feature of this class is that they have a tyrosine (or occasionally isoleucine) at the +2 position and a small residue at the +3 position. Finally, class IV peptides have a consensus sequence of (I/V/L)XpY(F/M)XP (Table 2). Interestingly, although the class III and IV peptides were less abundant overall, they were disproportionately represented among the most intensely colored beads (boldfaced sequences in Table 2). Class III and IV peptides bind to the SHP-2 N-SH2 domain with exceptionally high affinity (vide infra).

Specificity of the SHIP SH2 Domain. The SH2 domain from SHIP was screened against the pY library at two different SH2 protein concentrations (10 and 50 nM), and the peptide sequences from the 158 intensely colored beads were determined (Table 3). The SHIP SH2 domain recognizes a single consensus of pY(Y/S/T/v)(L/y/nle/f)(L/Nle/i/v) (Figure 4). Its specificity overlaps with those of SHP SH2 domains but also has a number of unique features. First, on the N-terminal side of pY, the SHIP SH2 domain does not require specific residues for high-affinity binding, although among the selected sequences there appears to be a higher than expected number of small residues (e.g., Gly, Pro, and Ala) at the −2 and −1 positions. Second, high-affinity binding to the SHIP SH2 domain requires a hydrophobic residue at the +2 position, with leucine being the most preferred, followed by tyrosine, norleucine, and phenylalanine. The latter feature had previously been noted by other investigators (*26*). Third, while alanine is among the most preferred amino acids at the +1 position for SHP SH2 domains, it is not favored by the SHIP SH2 domain. Among the nearly 200 SHIP SH2-binding sequences selected from the pY library (Table 3 and Table S3 in Supporting Information), only two had an alanine at the +1 position (TMpYAFI and TVpYALM).

Comparison with Previous Method: Specificity of the SHP-1 C-SH2 Domain. We have previously determined the

Table 3: pY Peptides Selected against the SHIP SH2 Domain (158 Total)[a]

TMpYAFI	FApYSYL	VYpYYYL	AGpYVFM
PYpYSFI	VYpYSYL	IKpYYYL	DGpYVLM
PFpYSFI	NYpYSYL	FMpYYYL	HPpYVLM
PApYSMI	**VYpYTLL**	QTpYFLM	PLpYVLM
PFpYSVI	TGpYTLL	SGpYFLM	YDpYVLM
PApYSYI	IQpYTLL	YGpYFLM	LGpYVMM
VYpYSYI	YMpYTLL	PPpYSFM	WApYVMM
AYpYSYI	FFpYTLL	AMpYSFM	EGpYVYM
TYpYTFI	SSpYTLL	YQpYSIM	VGpYVYM
PYpYTLI	NGpYTLL	**FVpYSLM**	GGpYYFM
PFpYTLI	NMpYTLL	YFpYSLM	EGpYYFM
GYpYTLI	GApYVFL	PPpYSMM	**GTpYYLM**
GPpYTLI	**VGpYVLL**	YGpYSMM	GYpYYLM
GGpYTMI	SLpYVLL	QGpYSMM	**AVpYYLM**
YYpYTYI	HKpYVLL	PMpYSTM	AHpYYLM
VGpYYFI	QGpYVLL	**PRpYSTM**	TRpYYLM
PGpYYLI	TSpYVLL	PRpYSVM	MQpYYLM
TGpYYLI	VSpYVLL	PRpYSYM	FKpYYLM
AGpYYMI	MGpYVML	VYpYTLM	HPpYYLM
VGpYYMI	QGpYVML	PMpYTLM	SApYYMM
FNpYYMI	WGpYVML	PRpYTLM	VTpYYMM
GGpYYVI	**AYpYYLL**	LPpYTLM	MKpYYMM
SFpYYYI	PIpYYLL	**YVpYTLM**	GIpYYYM
TSpYYYI	TGpYYLL	YGpYTLM	**ATpYYYM**
PFpYFLL	TGpYYLL	FTpYTLM	TGpYYYM
TVpYFLL	VGpYYLL	MTpYTLM	AGpYFYV
KGpYQLL	VKpYYLL	AHpYTLM	PRpYSLV
TMpYSFL	LGpYYLL	SApYTLM	VYpYSLV
PLpYSIL	MGpYYLL	SPpYTLM	PKpYSYV
AVpYSIL	FNpYYLL	HPpYTLM	YApYSYV
PFpYSLL*	FYpYYLL	HYpYTLM	YLpYSYV
HSpYSLL	WYpYYLL	RWpYTLM	AGpYYFV
LYpYSLL	HPpYYLL	WLpYTLM	AYpYYLV
VLpYSLL	QIpYYLL	TIpYTMM	WVpYYLV
VYpYSLL	LQpYYML	FQpYTMM	SApYYYV
TLpYSLL	FApYYML	GGpYTMM	SYpYYYV
RGpYSML	YGpYYML	ALpYTMM	LLpYYYV
PLpYSTL	NApYYML	QYpYTMM	MVpYYYV
TYpYSVL	GIpYYYL	FTpYTYM	
PKpYSYL	TTpYYYL	STpYTYM	

[a] Boldfaced sequences were from the most intensely colored beads from 10 nM SH2 domain screening; the sequence with an asterisk was selected for SPR analysis; M = norleucine.

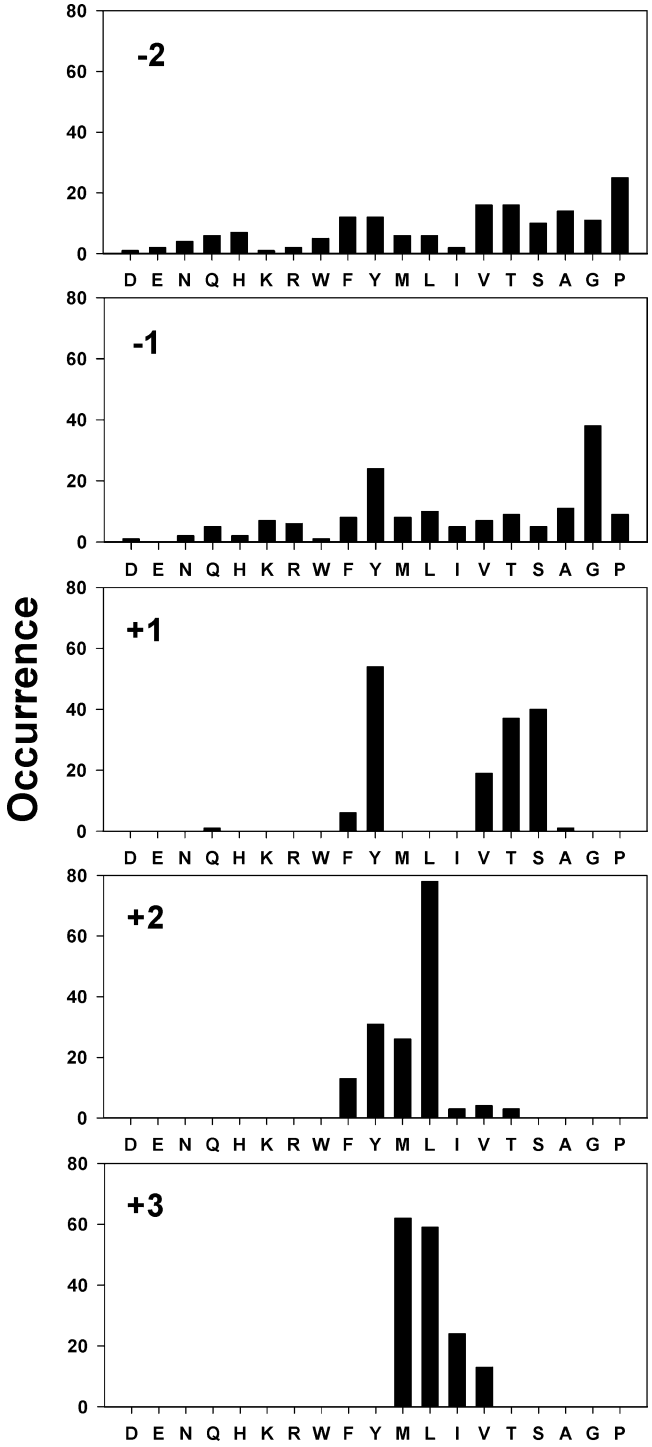

FIGURE 4: Specificity of SHIP SH2 domain. Displayed are the amino acids identified at each position (−2 to +3). Occurrence on the *y* axis represents the number of selected sequences that contain a particular amino acid at a certain position. Key: M, norleucine.

sequence specificity of SHP-1 N- and C-SH2 domains using a different method (*15*). In our previous method, the full-length peptide on a bead was encoded by generating a series of sequence-specific chain-termination products (a peptide ladder) during library synthesis (*27*). The sequence of the full-length peptide was determined through MALDI-TOF analysis of the peptide ladder in a manner similar to that described above. We felt that the previous method might bias library screening against sequences that contain slow-coupling amino acids (e.g., threonine and valine). Because

Table 4: pY Sequences Selected against the SHP-1 C-SH2 Domain (95 Total)[a]

VApYACL	TApYCML	IRpYASM	TRpYTRV
VYpYACL	ACpYCML	YWpYATM	YCpYTTV
IKpYAKL	TEpYCML	TQpYAVM	TRpYTTV
TQpYAML	VEpYCML	IRpYAVM	TQpYTRI
HRpYAML	SHpYCML	HRpYCKM	AIpYTLC
YYpYAQL	THpYCML	ALpYCLM	
TApYARL	IKpYCQL	TEpYCRM	SRpYHWF
HHpYARL	TSpYCQL	VVpYCRM	MHpYWRF
FRpYARL	QHpYCRL	TQpYCWM	TVpYSNK
YWpYARL	VHpYCRL	TRpYCWM	WWpYRVN
HVpYATL	TNpYCRL	TRpYCWM	VFpYHPQ
TApYAVL	VNpYCRL	TApYTCM	IWpYHPQ
TApYAVL	AApYCVL	TYpYTRM	YApYNFR
SHpYAVL	AApYCVL	VApYAVV	GHpYTFR
TQpYAVL	CApYCVL	TApYCCV	YHpYWQR
VHpYCCL	TApYCVL	TQpYCLV	YSpYYAR
TCpYCEL	TApYCVL	MYpYCRV	FTpYYAR
ACpYCIL	TFpYTAL	SCpYCSV	MYpYYNR
YRpYCKL	TIpYTTL	TYpYSCV	YGpYRYS
IApYCLL	IApYACM	AMpYSLV	YRpYFQY
VNpYCLL	YNpYAKM	VTpYTKV	WYpYKRY
TTpYCLL	TQpYAKM	VMpYTLV	TVpYRFY
VTpYCNL	VApYAMM	IMpYTNV	KRpYWFY
HApYCML	VRpYAMM	ITpYTQV	MFpYYRY

[a] Sequences were obtained from two screening experiments performed at 10 nM SHP-1 C-SH2 domain. C = α-aminobutyric acid; M = norleucine.

of their *β*-branching and bulky side-chain protecting group (*tert*-butyl for threonine), Fmoc-Thr(tBu)-OH and Fmoc-Val-OH are hindered and react slowly during coupling, resulting in a higher percentage of chain termination (reaction with Ac-Gly) and smaller amounts of full-length peptides on the bead surface. To test this notion and to demonstrate the advantage of our current method, we rescreened another pY library, which includes α-aminobutyric acid (Abu) as a cysteine surrogate at the random positions but is otherwise identical to the pY library described above, against the C-SH2 domain of SHP-1 (10 nM). Out of a total of 95 sequences obtained from 150 mg of resin, 77 belong to the class with a consensus sequence of (T/v/i)XpY(Abu/A/t)X-(L/m/v) (Table 4 and Figure 5). This is in general agreement with the previous result (*15*), but with a few subtle differences. The most notable difference is at the −2 position. The previous study suggested valine, isoleucine, leucine, and tyrosine as the most preferred residues, whereas the current data show that threonine is the most preferred amino acid, followed by valine and isoleucine (Figure 5). At positions +1 and +2, the current method also selected a larger number of threonine residues as compared to the previous method. The simplest explanation for the observed discrepancy is that the previous method biased against threonine-containing sequences. At the +1 position, the previous study produced almost exclusively alanine, whereas the current work shows that Abu is the most preferred amino acid, followed by alanine and threonine. Note that the previous library did not contain Abu at the random positions (*15*). The remaining 18 sequences do not show a clear consensus (Table 4). Some of the sequences were likely selected due to their binding directly to SA-AP (e.g., pYHPQ) (*16*), whereas others show some resemblance to the type II sequences previously selected against the SHP-1 C-SH2 domain (e.g., pYYXR) (*15*). Further work is underway to assess whether some of these peptides actually bind to the C-SH2 domain.

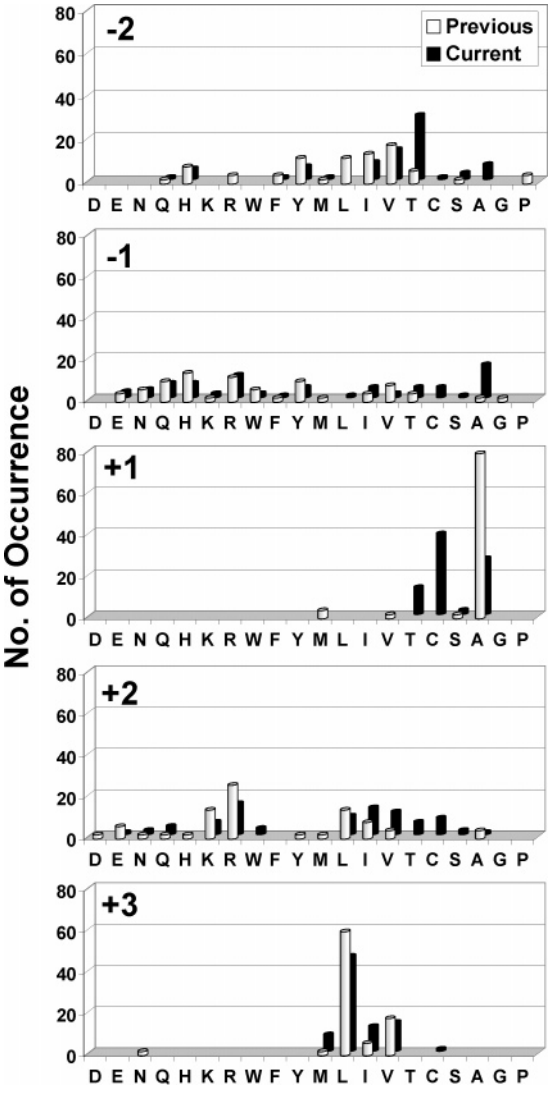

FIGURE 5: Comparison of the specificity of the SHP-1 C-SH2 domain as determined by the previous (open bar) vs current method (closed bar). Displayed are the amino acids identified at each position (−2 to +3). Number of occurrence on the *y* axis represents the number of selected sequences that contain a particular amino acid at a certain position (the previous *y* axis values were amplified by a factor of 2 to facilitate comparison). Key: C, aminobutyric acid; M, norleucine.

Affinity Measurements of Selected Sequences. To verify the screening results, representative peptides from each consensus group (peptides labeled with asterisks in Tables 1−3) were individually synthesized and tested for binding to the five SH2 domains of SHP-1, SHP-2, and SHIP using the surface plasmon resonance (SPR) technique (BIAcore) (Figure 6). Two SHP-2 C-SH2 domain-binding peptides (TIpYATI and NApYATI), which were derived from intensely and lightly colored beads, respectively, were chosen to test whether the color intensity of a bead during screening correlates with the binding affinity of the peptide it carries. A predicted tight binding sequence was selected for the SHIP SH2 domain (PFpYSLL). For the SHP-2 N-SH2 domain, a total of 10 representative sequences with at least two from each class (both predicted tight and weaker binding sequences) were chosen for further analysis. A total of 65 equilibrium dissociation constants (K_D) were measured and summarized in Table 5. These data allow us to draw the

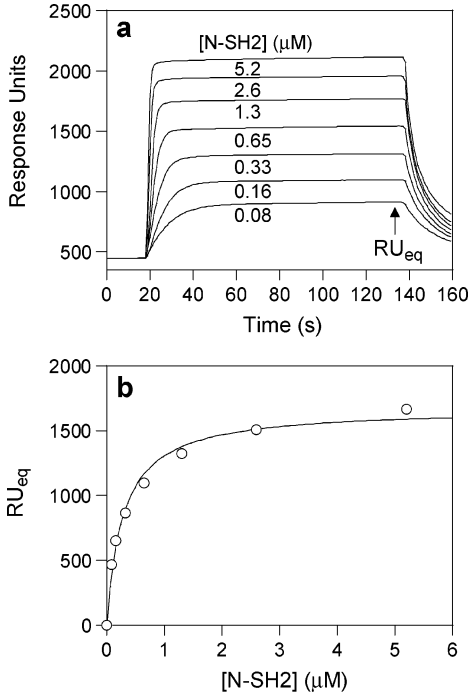

FIGURE 6: SPR analysis of the binding of the SHP-2 N-SH2 domain to peptide IHpYLYA. (a) Overlaid sensorgrams at indicated concentrations of N-SH2 protein (0.08−5.2 μM). (b) Plot of resonance signal under equilibrium binding conditions against SH2 concentration. The data were fitted to the equation $RU_{eq} = RU_{max}[SH2]/(K_D + [SH2])$.

following conclusions. First, all of the tested pY peptides bind to their cognate SH2 domains with high affinity (K_D = 0.044−9.7 μM) and have, in general, the highest affinity to the SH2 domain used in their selection. For example, peptide VIpYFVP was selected by the N-terminal SH2 domain of SHP-2 (class IV). It binds exceptionally tightly to the N-SH2 domain (K_D = 0.044 μM) but interacts with the other four SH2 domains with 23−360-fold lower affinity. Likewise, peptide PFpYSLL binds to the SHIP SH2 domain (which selected the former in the screening) with high affinity (K_D = 0.20 μM) but much less tightly to the other four SH2 domains (K_D = 5.6−14 μM). A few peptides (e.g., LVpYATI), however, can associate with all five SH2 domains with similar K_D values (2−5 μM), consistent with the previous observation that the five SH2 domains have overlapping sequence specificities. Second, for peptides within the same consensus group, there is a general correlation between bead color intensity and binding affinity (e.g., compare TIpYATI vs NApYATI for the C-SH2 domain and WMpYRII vs WTpYQIL, IHpYLYA vs ITpYTYP, and VIpYFVP vs VLpYMQP for the N-SH2 domain). The only exception is VApYVEL, which was derived from an intensely colored bead but binds SHP-2 N-SH2 domains with slightly lower affinity than LVpYATI and LVpYTEV (both from medium-colored beads). For peptides in the different classes, this correlation may not exist. For example, peptide VLpYMQP, a class IV peptide from a medium-colored bead, has a much higher affinity for the SHP-2 N-SH2 domain than class II peptide WMpYRII, which was from an intensely colored bead. This is because the color intensity of a bead is determined not only by the equilibrium K_D value but also by the kinetics of association and dissociation and possibly other factors. Peptides from different classes may have

Table 5: Dissociation Constants (μM) of Selected pY Peptides toward SHP-1, SHP-2, and SHIP SH2 Domains[a]

	SHP-2		SHP-1		
	N-SH2	C-SH2	N-SH2	C-SH2	SHIP SH2
(1) **TIpYATI**	3.9 ± 0.3	0.60 ± 0.07*	6.4 ± 0.4	2.4 ± 0.1	2.2 ± 0.2
(2) *NApYATI*	34 ± 3	3.9 ± 0.4*	28 ± 3	16 ± 2	10 ± 0.5
(3) **PFpYSLL**	9.7 ± 0.9	9.2 ± 1.5	5.6 ± 0.5	14 ± 4	0.20 ± 0.03*
(4) LVpYATI	1.9 ± 0.1*	2.0 ± 0.2	1.6 ± 0.5	5.2 ± 0.5	3.5 ± 0.2
(5) LVpYTEV	1.4 ± 0.1*	8.5 ± 1.2	3.2 ± 0.2	8.8 ± 1.3	3.8 ± 0.3
(6) **VApYVEL**	3.6 ± 0.1*	3.7 ± 0.1	4.9 ± 0.1	10 ± 1	5.2 ± 0.2
(7) LNpYMTF	2.4 ± 0.2*	8.9 ± 0.8	2.7 ± 0.2	>50	6.7 ± 0.9
(8) WTpYQIL	9.7 ± 0.3*	10 ± 1	17 ± 1	59 ± 4	3.8 ± 0.3
(9) **WMpYRII**	3.0 ± 0.4*	20 ± 6	8.5 ± 1.0	23 ± 5	6.3 ± 0.4
(10) **IHpYLYA**	0.28 ± 0.04*	12 ± 3	7.0 ± 0.4	27 ± 7	13 ± 1
(11) ITpYTYP	2.4 ± 0.2*	9.7 ± 1.9	2.1 ± 0.1	11 ± 3	3.2 ± 0.2
(12) VLpYMQP	0.11 ± 0.01*	4.9 ± 0.7	3.9 ± 0.2	>16	9.0 ± 0.9
(13) **VIpYFVP**	0.044 ± 0.014*	11 ± 2	1.0 ± 0.1	>16	3.8 ± 0.4

[a] The reported errors represent uncertainties from nonlinear regression fitting. For most of the interactions, at least two independent sets of measurements were performed to ensure the reproducibility of the measurements. All pY peptides are N-terminally acetylated and contain a C-terminal linker, LNBKR-NH_2. The lysine side chain was acylated with a PEG_4-biotin moiety for immobilization. SH2 domains were constructed as N-terminal six-histidine fusion proteins. Key: M, norleucine; bold type, peptides from most intensely colored beads; italic type, peptides from lightly colored beads. The asterisk indicates the SH2 domain by which each peptide was selected.

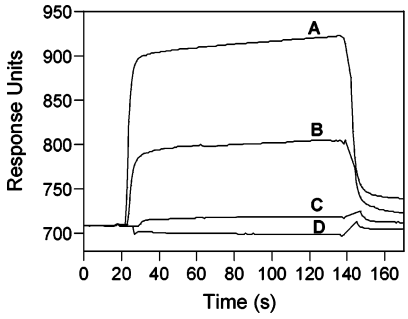

FIGURE 7: Competition of pY peptides for binding to the SHP-2 N-SH2 domain. Biotinylated peptide IHpYLYA was immobilized onto a sensorchip, and the N-SH2 domain in HBS-EP buffer (pH 7.4) was flowed over the chip surface at a flow rate of 15 μL/min. Key: (A) 0.3 μM SHP-2 N-SH2 protein alone; (B) 0.3 μM N-SH2 + 100 μM WMpYRII; (C) 0.3 μM N-SH2 + 100 μM LNpYMTF; (D) 0.3 μM N-SH2 + 100 μM LVpYTEV.

different binding modes and kinetics. For example, SPR analyses showed that the SHP-2 N-SH2 domain dissociated more slowly from immobilized peptides IHpYLYA and WMpYRII than peptide TIpYATI (data not shown). For peptides with similar K_D values, those with slower dissociation rates are expected to produce higher color intensity.

Competition Binding Experiments. To determine whether the four classes of pY peptides all bind to the same site on the SHP-2 N-SH2 domain surface, the peptides were subjected to binding competition to the SH2 domain by SPR. Peptide IHpYLYA (class III) was immobilized onto a sensorchip, and the N-SH2 protein was flowed over the chip surface. In the absence of competitor peptide, the N-SH2 domain (0.3 μM) bound to the immobilized IHpYLYA peptide, resulting in a large increase in resonance units (RU) (tracing A in Figure 7). However, when the N-SH2 domain (0.3 μM) was preincubated with 100 μM peptide LNpYMTF or LVpYTEV (class I), the binding was completely abolished (tracings C and D). Peptide WMpYRII (class II) reduced the amount of the N-SH2 domain bound to the sensorchip (tracing B), although it is less effective than the class I peptides. Peptides VLpYMQP and VIpYFVP (class IV) also effectively competed with the immobilized IHpYLYA for binding to the N-SH2 domain (data not shown). These results suggest that all four classes of pY peptides bind to the same site on the SH2 domain surface.

Binding of Selected pY Peptides to Intact SHP-1, SHP-2, and SHIP. To determine whether the pY peptides selected from the combinatorial library are capable of binding to full-length SHP-1, SHP-2, and SHIP proteins, peptide pull-down assays were performed with crude cell lysates derived from a murine cell line (Raw 264.7). Peptide TIpYATI and a positive control peptide (pITIM) from FcγRIIb (biotin-aminohexanoyl-EAENTITpYSLLKH-NH_2) (*14*) effectively precipitated both SHP-1 and SHP-2 from the cell lysate (Figure 8a). Peptide LVpYATI also precipitated SHP-1 and SHP-2 but was less effective. A negative control (G4), which is derived from the phosphorylated ITAM on the Fc receptor γ-subunit (biotin-aminohexanoyl-LLPDQLpYQPLKDRED-DQpYSHLQ-NH_2) (*28*), did not pull down any of the proteins. Surprisingly, peptides IHpYLYA and WMpYRII, which are selective ligands for the SHP-2 N-SH2 domain, failed to precipitate SHP-2 from the lysate. We noted that, in some experiments, these two peptides precipitated small amounts of proteins of lower molecular masses (data not shown). We reasoned that the N-SH2 domain-specific peptides might have disengaged the intramolecular association between the N-SH2 domain and the PTP domain, resulting in the SHPs in their open, active conformation (*29*, *30*), which were presumably cleaved into smaller species by protease(s) during the overnight incubation. Addition of common serine protease inhibitors did not prevent proteolysis. To this end, the pull-down assays were repeated with purified recombinant full-length SHP-1 and SHP-2. All of the tested pY peptides precipitated SHP-2, although IHpYLYA was less effective than the other peptides (Figure 8b). The lower effectiveness of IHpYLYA is most likely due to its marginal aqueous solubility. The pY peptides also precipitated SHP-1, albeit less effectively. None of the peptides bound to SHIP. These results demonstrate that the pY peptides selected against the isolated SH2 domains are indeed capable of selectively binding to the corresponding intact proteins.

Database Search of Potential SHP-1/SHP-2-Binding Proteins. The SH2 domain consensus sequences were employed

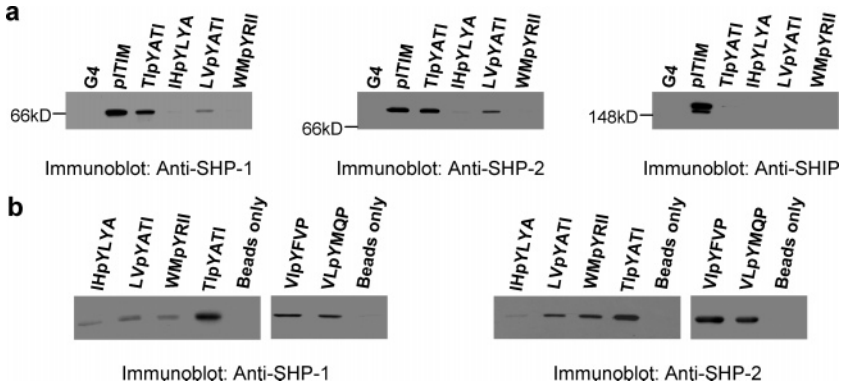

FIGURE 8: Pull-down assay of the interactions between pY peptides and full-length SHP-1, SHP-2, and SHIP. Biotinylated pY peptides were incubated with crude cell lysate (panel a) or purified protein (panel b). The peptide−protein complexes were captured by streptavidin−agarose beads and analyzed by western blotting. Key: G4, an ITAM peptide (negative control); pITIM, an ITIM peptide (positive control); beads only, no pY peptide added.

to search protein databases for potential SHP-1/2-binding proteins at the Protein Information Resource (PIR; web site, http://pir.georgetown.edu/). Since our initial searches with a single consensus motif resulted in a large number of "hits" (usually a few thousand) and both N- and C-SH2 domains of SHP-1/2 are often engaged in simultaneous interaction with tandem pY motifs, we narrowed our search using a tandem consensus sequence motif, [VIL]XY[ASTVI]X[ILV]X_{1-50}[TVIY]XY[ASTV]X[IVL], where X is any amino acid and the letters in brackets indicate the amino acids allowed at a given position. The two individual motifs were designed to encompass the N- (class I) and C-SH2 domain consensus sequences of both SHP's and were separated by anywhere from 1 to 50 residues. Any protein containing a sequence stretch that matches the tandem motif is considered as a positive "hit". A search of the human proteins resulted in 420 hits, representing ~100 unique proteins (many proteins appeared multiple times under different names or as fragments). After removing those proteins that we thought as "obvious" false positives (e.g., secreted proteins or transmembrane proteins with the consensus motifs in the extracellular environment), we obtained 77 proteins as potential SHP-1/SHP-2 targets (Table 6). Out of the 77 candidate proteins, 26 proteins have previously been shown to bind to SHP-1 and/or SHP-2 in a pY-dependent manner (*31*−*62*).

A literature search revealed that a total of 68 human proteins have previously been shown to interact with the SH2 domain(s) of SHP-1 and/or SHP-2 in the pY-dependent manner (Table 6 and Table S4 in Supporting Information). Thus, the above search has failed to identify the other 42 SHP-1/2-binding proteins. Sequence analysis of these proteins shows that 37 of these proteins contain one or more ITIM or ITIM-like motifs. The majority of them bind to SHP-1 and/or SHP-2 through a single ITIM or ITIM-like motif [e.g., CD72 (*63*, *64*), death receptor (*65*), epidermal growth factor receptor (*66*), erythropoietin receptor (*67*−*69*), leptin receptor (*70*), PD-1 (*71*, *72*), and platelet-derived growth factor receptor (*73*)]. Other proteins contain two or more ITIM motifs, but with one or more of their ITIM motifs containing a nonoptimal amino acid(s) at a critical position. For example, Gab-1, Gab-2, and Gab-3 each bind to SHP-2 via two ITIM motifs, [V/L]XpYLXL and VDpYVXV (*74*−*76*). The first ITIM motif has a leucine at the +1 position, which is an infrequently selected amino acid for SHP-2 SH2 domains (Figures 2 and 3) and was not included in the tandem motif used in the above database search. When a single consensus motif, [VIL]XY[ASTVI]X[ILV], was used to search the human protein database, 24 out of the 37 ITIM-containing proteins were recovered among the hits, in addition to the 26 proteins identified from the first search. Therefore, our searches against the class I consensus sequence identified 50 out of the 68 known SHP-1/2 targets (74%). Five of the 42 proteins are known to interact with SHP-1 and/or SHP-2, but not through classical ITIM motifs. These include c-Kit (YVpYIDP) (*77*), CTLA-4 (QPpYFIP) (*78*), E-selectin (GSpYQKP) (*79*), prolactin receptor (LD-pYLDP) (*80*), and STAT3 (putatively LVpYLYP) (*81*). Note that these motifs are very similar to the class III (pYLYP) and IV sequences (pYIDP, pYLDP, pYFIP, pYQKP) selected against SHP-2 N-SH2 domains (Table 2). These peptides can also bind tightly to the SHP-1 N-SH2 domain (Table 5) and indeed appeared among the class I sequences selected by the N-SH2 domain of SHP-1 (*15*).

DISCUSSION

The combinatorial library method reported in this work has for the first time provided a *complete* solution to the problem of identifying linear peptide motifs that interact with a given protein or nonprotein receptor. Compared to previously reported methods, our method has many significant advantages. First, our method identifies individual binding sequences; this feature is crucial for understanding the specificity of receptors that recognize multiple consensus sequences. For example, when the four classes of binding sequences of the SHP-2 N-SH2 domain were combined and plotted in the same manner as in Figure 2 to give a composite histogram, the specificity pattern was dominated by class I sequences (see Figure S1 in Supporting Information). It was impossible to winnow out the less abundant class III and IV sequences from the histogram, although they bind to the N-SH2 domain with higher affinity than class I peptides. Even for a receptor that has a single consensus sequence, individual sequences are useful in revealing the subtle covariance of sequences. For example, among the pY peptides that bind to the SHP-2 C-SH2 domain, when the +3 residue is isoleucine, alanine is most frequently found at the +1 position; however, when valine is the +3 residue, a serine is most preferred at the +1 position (Table 1). Second, our method allows for "fair" competition among all

Table 6: Human Proteins Predicted To Bind to SHP-1 and/or SHP-2 via SH2 Domains

protein	binding motif(s)	ref
activating NK cell receptor 2B4[b]	TIYSMI, TLYSLI	*31*
adenylate cyclase, type VI	VSYVVL, IAYTLL	
adipocyte G protein-coupled receptor 175	LVYSLV, YVYAGI	
alternative splicing factor-1	VCYADV, TAYIRV	
alternative splicing factor-3	VCYADV, VGYTRI	
B and T lymphocyte attenuator[a,b]	LLYSLL, IVYASL, TEYASI	*34*
β-hexosaminidase β-subunit	IEYARL, TTYSFL	
biliary glycoprotein-1 (CD66, CEACAM-1)[a,b]	VTYSTL, IIYSEV	*33*
coagulation factor II (thrombin) receptor	VCYVSI, VHYSFL, YVYSIL	
dol-P-man-dependent $\alpha(1-3)$-mannosyltransferase	VAYTEI, YDYTQL	
Ewing's sarcoma protein-1	LVYTSI, YPYSVL	
exportin-7, ran-binding protein-16	IGYSSV, TFYTAL, SYYSLL	
G protein-coupled receptor RDC1	VLYSFI, TEYSAL	
G6b-B protein of MHC III[a,b]	LLYADL, TIYAVV	*35*
H-rev107-like protein (HRLP-5)	VKYSRL, VQYSLI	
human germinal-center-associated lymphoma protein	LCYTLI, TEYSLL	
immune receptor expressed on myeloid cells-1 (polymeric immunoglobulin receptor)[a]	LCYADL, VEYVTM, ISYASL, TEYSTI	*36*, *37*
immunoglobulin superfamily receptor translocation associated-1 (IFGP-2)	LVYSEI, VVYSEV	
immunoglobulin superfamily receptor translocation associated-2	VVYSEV, IIYSEV	
immunoglobulin-like transcript 2, leukocyte immunoglobulin-like receptor-1 (MIR-7)[a]	VTYAEV, VTYAQL	*38*
immunoglobulin-like transcript 3 (LIR-5)[a]	VTYAKV, VTYAQL	*39*
immunoglobulin-like transcript 5 (LIR-3)	VTYAPV, VTYAQL	
inhibitory receptor protein 60 (IRC-1)[a,b]	LHYANL, VEYSTV, LHYASV	*40*
interleukin 8 receptor α (CXCR-1)	IAYALV, ILYSRV, IIYAFI	
interleukin 8 receptor β (CXCR-2)	IIYALV, ILYSRV, LIYAFI	
killer cell Ig-like receptor 2DL1 (p58, NKAT-1)[a,b]	VTYTQL, IVYTEL	*41*, *42*
killer cell Ig-like receptor 2DL2 (NKAT-6)	VTYTQL, IVYAEL	
killer cell Ig-like receptor 2DL3 (NKAT-2)	VTYAQL, IVYTEL	
killer cell Ig-like receptor 3DL1 (p70, NKB-1, NKAT-3)[a,b]	VTYAQL, ILYTEL	*43*
leucine-rich neuronal protein (LRCH-4)	VFYVVL, VTYTRL	
leukocyte antigen (CD84)[a,b]	TIYTYI, TVYSEV	*44*
leukocyte-associated immunoglobulin-like receptor-1[a,b]	VTYAQL, ITYAAV	*45*
lipid phosphate phosphorylase-1 (phosphatidic acid phosphatase-2a)	LPYVAL, IPYALL	
metabotropic glutamate receptor-2	LCYILL, VCYSAL	
metabotropic glutamate receptor-3	LCYILL, ICYSAL	
metabotropic glutamate receptor-4	LSYVLL, ISYAAL	
metabotropic glutamate receptor-7	LSYVLL, ISYAAL	
multiple C2-domain and transmembrane region protein-2	LRYIIL, VQYAEL	
natural killer inhibitory receptor NKG2-A[a,b]	VIYSDL, ITYAEL	*46*
natural killer-,T-, B-cell antigen receptor[a,b]	LEYVSV, TVYASV, TIYSTI	*47*
neuropeptides B/W receptor type 1 (GPR7)	VVYAVI, VLYVLL	
novel protein similar to PRAME	LSYVLL, IHYSQL	
olfactory receptor 1F1	LFYSTI, VLYTVV	
olfactory receptor 8D1	ILYSIL, VFYTTV	
olfactory receptor 12D2	LRYTVI, LFYAPV, IMYTVV	
olfactory receptor 12D3	ISYSSV, LRYTVI, IMYSAV	
olfactory receptor 51B5	ISYVLI, VFYVTV	
olfactory receptor 51V1	TVYTVL, LRYSSI	
osteoblast-specific factor-2	IKYIQI, IKYTRI	
paired immunoglobin-like type 2 receptor α (FDF03)[a,b]	IVYASL, TLYSVL	*48*, *49*
phosphoribosyl transferase domain containing-1	LEYVLI, IGYSDI	
PIG-M mannosyltransferase	VRYTDI, YRYTPL	
platelet endothelial cell adhesion molecule-1 (CD31)[a,b]	VQYTEV, TVYSEV	*50*
polycystin-1, polycystic kidney disease-related protein-1	VTYTPV, VQYVAL, LNYTLL	
protein KIAA0319 (contains polycystic kidney disease 1 domains)	IFYVTV, TKYTIL	
protein zero related[b]	VIYAQL, VVYADI	*51*, *52*
R3H domain protein-1	IPYTSV, VYYSVI	
ran-binding protein-17	VGYILL, TFYTAL, TSYTML, ICYSAL	
SH2 domain-containing phosphatase anchor protein-1[a]	VVYSQV, VIYSSV	*53*
sialic acid binding Ig-like lectin-2 (CD22)[a]	VTYSAL, IHYSEL, VDYSEL	*54*, *55*
sialic acid binding Ig-like lectin-3 (CD33)[a,b]	LHYASL, TEYSEV	*56*
sialic acid binding Ig-like lectin-5 (OBBP-2)	LHYASL, TEYSEI	
sialic acid binding Ig-like lectin-6 (OBBP-1)	LHYAVL, TEYSEI	
sialic acid binding Ig-like lectin-9 (FOAP-9)[a]	LQYASL, TEYSEI	*57*
sialic acid binding Ig-like lectin-11[a,b]	LHYASL, TEYSEI	*32*
sialic acid binding Ig-like lectin-12 (S2V)[a,b]	IQYASL, YEYSEI	*58*
signal regulatory protein α-1 (SHPS-1, BIT, MyD-1, PTPNS-1)[a,b]	ITYADL, TEYASI, LTYADL	*59*, *60*
signaling lymphocytic activation molecule (CD150)[b]	TIpYAQV, TVpYASV	*61*
sodium channel type V α subunit (cardiac muscle α-subunit)	LNYTIV, IMYAAV, TTYIII, IEYSVL	
sodium channel type XI α subunit (peripheral nerve sodium channel 5, hNaN)	INYTII, IIYAAV, VSYIII, IKYSAL	
solute carrier family 19, member 3 (SLC19A3)	LNYVQI, VGYVKV	
somatostatin receptor 1[b]	VIYVIL, VLYTFL, LCYVLI	*62*
spastic ataxia of Charlevoix−Saguenay	IHYTLL, YTYAII	
trace amine receptor-5 (GPR102)	LTYSGA, ILYSKI	
ubiquitin-specific protease-9, X chromosome (DFFRX)	VMYANL, YQYAEL	
ubiquitin-specific protease-9, Y chromosome (DFFRY)	VMYANL, YQYAEL	
zinc finger protein 521	VGYTSV, VTYSCI	

[a] Proteins that have previously been shown to bind to SHP-1 via its SH2 domains. [b] Proteins that have previously been shown to bind to SHP-2 via its SH2 domains.

library peptides, as each bead contains roughly the same amount of peptide molecules (~100 pmol). This is not the case with pY peptide libraries displayed on a phage surface, because such libraries are biased against sequences that are poor substrates of the tyrosine kinases used to phosphorylate the phage (*10*, *11*). Youngquist et al. reported another method in which the peptide sequence on each bead is encoded by generating a set of chain-termination products during library synthesis, and the sequence of the full-length peptide is determined by mass spectrometric analysis of the set of chain-termination products (*27*). Unfortunately, due to different reactivities of the 20 amino acids, the amount of chain termination varies with peptide sequence. As a result, the amount of full-length peptide on each bead also varies, biasing the screening against peptides containing slow-coupling amino acids (e.g., Thr and Ile). Indeed, a comparison of the peptides selected by the Youngquist method vs the current method showed that the former caused an underrepresentation of Thr-containing sequences (Figure 5). Third, because our method employs chemically synthesized libraries, modified (e.g., pY) and/or unnatural amino acids (e.g., D-amino acids) can be easily incorporated into the libraries. Fourth, our method is high-throughput and cost-effective. By employing partial Edman degradation, we can routinely sequence 20−30 beads in an hour, at a cost of ~$0.50 per bead. Fifth, as demonstrated with all four SH2 domains from SHP-1, SHP-2, and SHIP, our method is highly reproducible. It is readily applicable to other protein or nonprotein receptors. We have recently applied this method to determine the sequence specificity of BIR domains, WW domains, and chromodomains (unpublished results). Finally, it is worth noting that abundance in the library does not correlate with high affinity, especially when comparing two different classes of peptides. In fact, the opposite trend appears to be true, as demonstrated here by the class I and IV sequences for the SHP-2 N-SH2 domain. Presumably, a high-affinity interaction requires a better fit between the protein and peptide structures, necessarily limiting the number of possible choices in the library. This further underscores the importance of obtaining individual binding sequences.

The five SH2 domains of SHP-1, SHP-2, and SHIP have overlapping specificities, and yet each domain possesses some unique features. There are major differences between SHP and SHIP SH2 domains. SHP SH2 domains require a hydrophobic residue at the −2 position, whereas the SHIP SH2 domain can tolerate most of the amino acids at the N-terminal side of pY. On the C-terminal side, SHIP SH2 strongly prefers a Leu at the +2 position, but SHP SH2 domains have no such requirement (except for class II peptides of the SHP-2 N-SH2 domain). There are also more subtle differences at the +1 position; while SHP SH2 domains all prefer an alanine at this position, alanine was seldom found at this position among all of the SHIP SH2-binding sequences. Among the four SHP SH2 domains, the two N-terminal SH2 domains have similar specificities, and the two C-SH2 domains are highly analogous to each other. Most of the class I and II peptides selected by SHP-2 N-SH2 also bind to the SHP-1 N-SH2 domain with similar affinities (Table 5). However, many of the class III and IV peptides (e.g., IHpYLYA, VLpYMQP, and VIpYFVP) show excellent selectivity for the SHP-2 N-SH2 domain. They bind to the SHP-1 N-SH2 domain with >20-fold lower affinities and with even lower affinity to the other three SH2 domains. The two C-SH2 domains have two subtle differences (Figures 2 and 5). At the +3 position, SHP-1 most prefers leucine, whereas SHP-2 prefers isoleucine. At the +1 position, SHP-2 accepts serine as the second most preferred residue, whereas serine is disfavored by SHP-1.

The SH2 specificity data should be very useful in providing a molecular basis for different functions of SHP-1, SHP-2, and SHIP in cell signaling. For example, immunoreceptor PD-1 has been shown to co-immunoprecipitate with endogenous SHP-2 but not SHP-1 (*71*, *72*). The pY motif responsible for SHP-2 binding is TEpYATIVF, which matches perfectly with the consensus sequence of the SHP-2 C-SH2 domain (Figure 2). Our data predict that it should bind only weakly to the SHP-1 C-SH2 domain, which only occasionally selected an isoleucine at the +3 position (Figure 5). Indeed, a synthetic peptide containing the TEpYATIVF motif bound SHP-2 much more strongly than SHP-1 in vitro (*72*). SHIP has been reported as the main inhibitory molecule for the immunoglobulin G Fc receptor signaling pathway by binding to the pYSLL motif on the Fc receptor (*26*, *82*). The pYSLL motif matches the consensus sequence of the SHIP SH2 domain and binds the SHIP SH2 domain with much higher affinity than the SH2 domains of SHP-1 or SHP-2 (Table 5). Many receptors, however, contain multiple ITIM motifs that match the specificities of both SHP-1 and SHP-2. For example, the first ITIM motif of human Siglec-11 (LHpYASL) closely matches the consensus sequence of SHP-1 SH2 domains, whereas its second ITIM, TEpYSEI, matches the consensus of the SHP-2 C-SH2 domain (*32*). Some receptors contain ITIM motifs whose sequences represent a compromise between the consensus of SHP-1 and SHP-2 SH2 domains. Biliary glycoprotein 1 (CD66), which is known to bind both SHP-1 and SHP-2, is such an example (*33*). Its two ITIM motifs (VTpYSTL and IIpYSEV) match the overlapping specificities of SHP-1 and SHP-2 SH2 domains.

The specificity data can also be used to predict the interaction partners of the SH2 domain-containing proteins. As described above, simple database searches have identified 74% of the known SHP-1 and SHP-2 interacting proteins (Table 6 and Table S4 in Supporting Information). It is highly probable that some of the other predicted proteins in Table 6 will prove to be bona fide SHP-1 and SHP-2 binding proteins. Although at the present time, database searches using a single consensus motif generate too many false positives, the number of false positives can be greatly reduced by applying additional constraints. One such constraint is tissue distribution and subcellular localization. Another restriction is phosphorylation, which is required for binding for the vast majority of SH2 domains. Databases on phosphorylation sites and kinase specificity are becoming increasingly available in recent years (web sites: http://www.cbs.dtu.dk/databases/PhosphoBase/; http://phospho.elm.eu.org/about.html). Finally, one can make educated guesses on the basis of the function of a protein. Indeed, many of the 68 SHP-1/2-binding proteins have been discovered by the presence of ITIM motif(s) in their sequences. Additionally, this work has uncovered a new recognition motif, [IVL]XpY[LMF]XP, for the N-SH2 domains of SHP-1 and SHP-2. It remains to be determined whether

nature has utilized the class II and other class III motifs for binding to SHP-1 and SHP-2.

In summary, a powerful combinatorial library method has now been developed for the systematic determination of sequence specificities of protein interaction domains such as SH2 domains. The specificity information generated by this method will be very useful in understanding the cellular function of proteins that contain these interaction domains and the design of specific inhibitors against such protein domains.

SUPPORTING INFORMATION AVAILABLE

Additional SH2 domain-binding sequences, comparison of specificities of SHP-1/2 and SHIP SH2 domains, and a list of known SHP-1/2-interacting proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Pawson, T., and Nash, P. (2003) Assembly of cell regulatory systems through protein interaction domains, *Science 300*, 445−452.
2. Venter, J. C., et al. (2001) The sequence of the human genome, *Science 291*, 1304−1351.
3. Bork, P., Schultz, J., and Ponting, C. P. (1997) Cytoplasmic signaling domains: the next generation, *Trends Biochem. Sci. 22*, 296−298.
4. 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) SH2 domains recognize specific phosphopeptide sequences, *Cell 72*, 767−778.
5. De Souza, D., Fabri, L. J., Nash, A., Hilton, D. J., Nicola, N. A., and Baca, M. (2002) SH2 domains from suppressor of cytokine signaling-3 and protein tyrosine phosphatase SHP-2 have similar binding specificities, *Biochemistry 41*, 9229−9236.
6. Muller, K., Gombert, F. O., Manning, U., Grossmuller, F., Graff, P., Zaegel, H., Zuber, J. F., Freuler, F., Tschopp, C., and Baumann, G. (1996) Rapid identification of phosphopeptide ligands for SH2 domains: Screening of peptide libraries by fluorescence-activated bead sorting, *J. Biol. Chem. 271*, 16500−16505.
7. Houghten, R. A., Pinilla, C., Blondelle, S. E., Appel, J. R., Dooley, C. T., and Cuervo, J. H. (1991) Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery, *Nature 354*, 84−86.
8. Scott, J. K., and Smith, G. P. (1990) Searching for peptide ligands with an epitope library, *Science 249*, 386−390.
9. Rickles, R. J., Botfield, M. C., Weng, Z., Taylor, J. A., Green, O. M., Brugge, J. S., and Zoller, M. J. (1994) Identification of Src, Fyn, Lyn, PI3K, and Abl SH3 domain ligands using phage display libraries, *EMBO J. 13*, 5598−5604.
10. Gram, H., Schmitz, R., Zuber, J. F., and Baumann, G. (1997) Identification of phosphopeptide ligands for the Src-homology 2 (SH2) domain of Grb2 by phage display, *Eur. J. Biochem. 246*, 633−637.
11. King, T. R., Fang, Y., Mahon, E. S., and Anderson, D. H. (2000) Using a phage display library to identify basic residues in A-Raf required to mediate binding to the Src homology 2 domains of the p85 subunit of phsophatidylinositol 3′-kinase, *J. Biol. Chem. 275*, 36450−36456.
12. Ganesan, L. P., Wei, G., Pengal, R. A., Moldovan, L., Moldovan, N., Ostrowski, M. C., and Tridandapani, S. (2004) The serine/threonine kinase Akt promotes Fcγ receptor-mediated phagocytosis in murine macrophages through the activation of p70S6 kinase, *J. Biol. Chem. 279*, 54416−54425.
13. Sugimoto, S., Lechleider, R. J., Shoelson, S. E., Neel, B. G., and Walsh, C. T. (1993) Expression, purification, and characterization of SH2-containing protein tyrosine phosphatase, SH-PTP2, *J. Biol. Chem. 268*, 22771−22776.
14. Tridandapani, S., Kelley, T., Pradhan, M., Cooney, D., Justement, L. B., and Coggeshall, K. M. (1997) Recruitment and phosphorylation of SHIP and Shc to the B cell Fc gamma ITIM peptide motif, *Mol. Cell. Biol. 17*, 4305−4311.
15. Beebe, K. D., Wang, P., Arabaci, G., and Pei, D. (2000) Determination of binding specificity of the SH2 domains of protein tyrosine phosphatase SHP-1 through the screening of a combinatorial phosphotyrosyl peptide library, *Biochemistry 39*, 13251−13260.
16. Lam, K. S., Salmon, S. E., Hersh, E. M., Hruby, V. J., Kazmierski, W. M., and Knapp, R. J. (1991) A new type of synthetic peptide library for identifying ligand-binding activity, *Nature 354*, 82−84.
17. Furka, A., Sebestyen, F., Asgedom, M., and Dibo, G. (1991) General method for rapid synthesis of multicomponent peptide mixtures, *Int. J. Pept. Protein Res. 37*, 487−493.
18. Sweeney, M. C., and Pei, D. (2003) An improved method for rapid sequencing of support-bound peptides by partial Edman degradation and mass spectrometry, *J. Comb. Chem. 5*, 218−222.
19. Neel, B. G., Gu, H., and Pao, L. (2000) The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling, *Trends Biochem. Sci. 28*, 284−293.
20. Ravetch, J. V., and Lanier, L. L. (2000) Immune inhibitory receptors, *Science 290*, 84−89.
21. Waksman, G., Kominos, D., Robertson, S. C., Pant, N., Baltimore, D., Birge, R. B., Cowburn, D., Hanafusa, H., Mayer, B. J., Overduin, M., Resh, M. D., Rios, C. B., Silverman, L., and Kuriyan. J. (1992) Crystal structure of the phosphotyrosine recognition domain SH2 of v-src complexed with tyrosine-phosphorylated peptides, *Nature 358*, 646−653.
22. Eck, M. J., Atwell, S. K., Shoelson, S. E., and Harrison, S. C. (1994) Structure of the regulatory domains of the Src-family tyrosine kinase Lck, *Nature 368*, 764−769.
23. Lee, C.-H., Kominos, D., Jacques, S., Margolis, B., Schlessinger, J., Shoelson, S. E., and Kuriyan, J. (1994) Crystal structures of peptide complexes of the amino-terminal SH2 domain of the Syp tyrosine phosphatase, *Structure 2*, 423−438.
24. Burshtyn, D. N., Yang, W., Yi, T., and Long, E. O. (1997) A novel phosphotyrosine motif with a critical amino acid at position −2 for the SH2 domain-mediated activation of tyrosine phosphatase SHP-1, *J. Biol. Chem. 272*, 13066−13072.
25. Liao, H., Yuan, C., Su, M.-I., Yongkiettrakul, S., Qin, D., Li, H., Byeon, I.-J. L., Pei, D., and Tsai, M.-D. (2000) Structure of the FHA1 domain of yeast Rad53 and identification of binding sites for both FHA1 and its target protein Rad9, *J. Mol. Biol. 304*, 941−951.
26. Bruhns, P., Vely, F., Malbec, O., Fridman, W. H., Vivier, E., and Daeron, M. (2000) Molecular basis of the recruitment of the SH2 domain-containing inositol 5-phosphatases SHIP1 and SHIP2 by FcγRIIB, *J. Biol. Chem. 275*, 37357−37364.
27. Youngquist, R. S., Fuentes, G. R., Lacey, M. P., and Keough, T. (1995) Generation and screening of combinatorial peptide libraries designed for rapid sequencing by mass spectrometry, *J. Am. Chem. Soc. 117*, 3900−3906.
28. Pengal, R. A., Ganesan, L. P., Fang, H., Marsh, C. B., Anderson, C. L., and Tridandapani, S. (2003) SHIP-2 inositol phosphatase is inducibly expressed in human monocytes and serves to regulate Fcγ receptor-mediated signaling, *J. Biol. Chem. 278*, 22657−22663.
29. Pei, D., Lorenz, U., Klingmuller, U., Neel, B. G., and Walsh, C. T. (1994) Intramolecular regulation of protein tyrosine phosphatase SH-PTP1: A new function for Src homology 2 domains, *Biochemistry 33*, 15483−15493.
30. Hof, P., Pluskey, S., Dhe-Paganon, S., Eck, M. J., and Shoelson, S. E. (1998) Crystal structure of the tyrosine phosphatase SHP-2, *Cell 92*, 441−450.
31. Tangye, S. G., Lazetic, S., Woollatt, T. E., Sutherland, G. R., Lanier, L. L., and Phillips, J. H. (1999) Cutting edge: Human 2B4, an activating NK cell receptor, recruits the protein tyrosine phosphatase SHP-2 and the adaptor signaling protein SAP, *J. Immunol. 162*, 6981−6985.
32. Angata, T., Kerr, S. C., Greaves, D. R., Varki, N. M., Crocker, P. R., and Varki, A. (2002) Cloning and characterization of human Siglec-11, *J. Biol. Chem. 277*, 24466−24474.
33. Huber, M., Izzi, L., Grondin, P., Houde, C., Kunath, T., Veillette, A., and Beauchemin, N. (1999) The carboxyl-terminal region of biliary glycoprotein controls its tyrosine phosphorylation and association with protein-tyrosine phosphatases SHP-1 and SHP-2 in epithelial cells, *J. Biol. Chem. 274*, 335−344.
34. Gavrieli, M., Watanabe, N., Loftin, S. K., Murphy, T. L., and Murphy, K. M. (2003) Characterization of phosphotyrosine binding motifs in the cytoplasmic domain of B and T lymphocyte

attenuator required for association with protein tyrosine phosphatases SHP-1 and SHP-2, *Biochem. Biophys. Res. Commun. 312*, 1236−1243.
35. De Vet, E. C. J. M., Aguado, B., and Campbell, R. D. (2001) G6b, a novel immunoglobulin superfamily member encoded in the human major histocompatibility complex, interacts with SHP-1 and SHP-2, *J. Biol. Chem. 276*, 42070−42076.
36. Sui, L., Li, N., Liu, Q., Zhang, W., Wan, T., Wang, B., Luo, K., Sun, H., and Cao, X. (2004) IgSF13, a novel human inhibitory receptor of the immunoglobulin superfamily, is preferentially expressed in dendritic cells and monocytes, *Biochem. Biophys. Res. Commun. 319*, 920−928.
37. Alvarez-Errico, D., Aguilar, H., Kitzig, F., Brckalo, T., Sayos, J., and Lopez-Botet, M. (2004) IREM-1 is a novel inhibitory receptor expressed by myeloid cells, *Eur. J. Immunol. 34*, 3690−3701.
38. Fanger, N. A., Cosman, D., Peterson, L., Braddy, S. C., Maliszewski, C. R., and Borges, L. (1998) The MHC class I binding proteins LIR-1 and LIR-2 inhibit Fc receptor-mediated signaling in monocytes, *Eur. J. Immunol. 28*, 3423−3434.
39. Cella, M., Dohring, C., Samaridis, J., Dessing, M., Brockhaus, M., Lanzavecchia, A., and Colonna, M. (1997) A novel inhibitory receptor (ILT3) expressed on monocytes, macrophages, and dendritic cells involved in antigen processing, *J. Exp. Med. 185*, 1743−1751.
40. Cantoni, C., Bottino, C., Augugliaro, R., Morelli, L., Marcenaro, E., Castriconi, R., Vitale, M., Pende, D., Sivori, S., Millo, R., Biassoni, R., Moretta, L., and Moretta, A. (1999) Molecular and functional characterization of IRp60, a member of the immunoglobulin superfamily that functions as an inhibitory receptor in human NK cells, *Eur. J. Immunol. 29*, 3148−3159.
41. Burshtyn, D. N., Scharenberg, A. M., Wagtmann, N., Rajagopalan, S., Berrada, K., Yi, T., Kinet, J.-P., and Long, E. O. (1996) Recruitment of tyrosine phosphatase HCP by the killer cell inhibitory receptor, *Immunity 4*, 77−85.
42. Olcese, L., Lang, P., Vely, F., Cambiaggi, A., Marguet, D., Blery, M., Hippen, K. L., Biassoni, R., Moretta, A., Moretta, L., Cambier, J. C., and Vivier, E. (1996) Human and mouse killer-cell inhibitory receptors recruit PTP1C and PTP1D protein tyrosine phosphatases, *J. Immunol. 156*, 4531−4534.
43. Fry, A. M., Lanier, L. L., and Weiss, A. (1996) Phosphotyrosines in the killer cell inhibitory receptor motif of NKB1 are required for negative signaling and for association with protein tyrosine phosphatase 1C, *J. Exp. Med. 184*, 295−300.
44. Lewis, J., Eiben, L. J., Nelson, D. L., Cohen, J. I., Nichols, K. E., Ochs, H. D., Notarangelo, L. D., and Duckett, C. S. (2001) Distinct interactions of the X-linked lymphoproliferative syndrome product SAP with cytoplasmic domains of members of the CD2 receptor family, *Clin. Immunol. 100*, 15−23.
45. Verbrugge, A., de Ruiter, T., Clevers, H., and Meyaard, L. (2003) Differential contribution of the immunoreceptor tyrosine-based inhibitory motifs of human leukocyte-associated Ig-like receptor-1 to inhibitory function and phosphatase recruitment, *Int. Immunol. 15*, 1349−1358.
46. Carretero, M., Palmieri, G., Llano, M., Tullio, V., Santoni, A., Geraghty, D. E., and Lopez-Botet, M. (1998) Specific engagement of the CD94/NKG2-A killer inhibitory receptor by the HLA-E class Ib molecule induces SHP-1 phosphatase recruitment to tyrosine-phosphorylated NKG2-A: Evidence for receptor function in heterologous transfectants, *Eur. J. Immunol. 28*, 1280−1291.
47. Bottino, C., Falco, M., Parolini, S., Marcenaro, E., Augugliaro, R., Sivori, S., Landi, E., Biassoni, R., Notarangelo, L. D., Moretta, L., and Moretta, A. (2001) NTB-A, a novel SH2D1A-associated surface molecule contributing to the inability of natural killer cells to kill Epstein−Barr virus infected B cells in X-linked lymphoproliferative disease, *J. Exp. Med. 194*, 235−246.
48. Mousseau, D. D., Banville, D., L'Abbe, D., Bouchard, P., and Shen, S. H. (2000) PILRα, a novel immunoreceptor tyrosine-based inhibitory motif-bearing protein, recruits SHP-1 upon tyrosine phosphorylation and is paired with the truncated counterpart PILR*β*, *J. Biol. Chem. 275*, 4467−4474.
49. Fournier, N., Chalus, L., Durand, I., Garcia, E., Pin, J.-J., Churakova, T., Patel, S., Zlot, C., Gorman, D., Zurawski, S., Abrams, J., Bates, E. E. M., and Garrone, P. (2000) FDF03, a novel inhibitory receptor of the immunoglobulin superfamily, is expressed by human dendritic and myeloid cells, *J. Immunol. 165*, 1197−209.
50. Wong, M. X., and Jackson, D. E. (2004) Regulation of B cell activation by PECAM-1: Implications for the development of autoimmune disorders, *Curr. Pharm. Des. 10*, 155−161.
51. Zhao, Z. J., and Zhao, R. (1998) Purification and cloning of PZR, a binding protein and putative physiological substrate of tyrosine phosphatase SHP-2, *J. Biol. Chem. 273*, 29367−29372.
52. Zhao, R., and Zhao, Z. J. (2000) Dissecting the interaction of SHP-2 with PZR, an immunoglobulin family protein containing immunoreceptor tyrosine-based inhibitory motifs, *J. Biol. Chem. 275*, 5453−5459.
53. Xu, M. J., Zhao, R., and Zhao, Z. J. (2001) Molecular cloning and characterization of SPAP1, an inhibitory receptor, *Biochem. Biophys. Res. Commun. 280*, 768−775.
54. Doody, G. M., Justement, L. B., Delibrias, C. C., Matthews, R. J., Lin, J., Thomas, M. L., and Fearon, D. T. (1995) A role in B cell activation for CD22 and the protein tyrosine phosphatase SHP, *Science 269*, 242−244.
55. Law, C.-L., Sidorenko, S. P., Chandran, K. A., Zhao, Z., Shen, S.-H., Fischer, E. H., and Clark, E. A. (1996) CD22 associates with protein tyrosine phosphatase 1C, Syk, and phospholipase C-*γ*1 upon B cell activation, *J. Exp. Med. 183*, 547−560.
56. Taylor, V. C., Buckley, C. D., Douglas, M., Cody, A. J., Simmons, D. L., and Freeman, S. D. (1999) The myeloid-specific sialic acid-binding receptor, CD33, associates with the protein-tyrosine phosphatases, SHP-1 and SHP-2, *J. Biol. Chem. 274*, 11505−11512.
57. Ikehara, Y., Ikehara, S. K., and Paulson, J. C. (2004) Negative regulation of T cell receptor signaling by siglec-7 (p70/AIRM) and siglec-9, *J. Biol. Chem. 279*, 43117−43125.
58. Yu, Z., Lai, C. M., Maoui, M., Banville, D., and Shen, S. H. (2001) Identification and characterization of S2V, a novel putative siglec that contains two V set Ig-like domains and recruits protein-tyrosine phosphatase SHPs, *J. Biol. Chem. 276*, 23816−23824.
59. Fujioka, Y., Matozaki, T., Noguchi, T., Iwamatsu, A., Yamao, T., Takahashi, N., Tsukda, M., Takada, T., and Kasuga, M. (1996) A novel membrane glycoprotein, SHPS-1, that binds the SH2-domain-containing protein tyrosine phosphatase SHP-2 in response to mitogens and cell adhesion, *Mol. Cell. Biol. 16*, 6887−6899.
60. Veillette, A., Thibaudeau, E., and Latour, S. (1998) High expression of inhibitory receptor SHPS-1 and its association with protein-tyrosine phosphatase SHP-1in macrophages, *J. Biol. Chem. 273*, 22719−22728.
61. Howie, D., Simarro, M., Sayos, J., Guirado, M., Sancho, J., and Terhorst, C. (2002) Molecular dissection of the signaling and costimulatory functions of CD150 (SLAM): CD150/SAP binding and CD150-mediated costimulation, *Blood 99*, 957−965.
62. Florio, T., Thellung, S., Arena, S., Corsaro, A., Bajetto, A., Schettini, G., and Stork, P. J. S. (2000) Somatostatin receptor 1 (SSTR-1)-mediated inhibition of cell proliferation correlates with the activation of the MAP kinase cascade: role of the phosphatase SHP-2, *J. Physiol. 94*, 239−250.
63. Wu, Y., et al. (1998) The B-cell transmembrane protein CD72 binds to and is an in vivo substrate of the protein tyrosine phosphatase SHP-1, *Curr. Biol. 8*, 1009−1017.
64. Adachi, T., Flaswinkel, H., Yakura, H., Reth, M., and Tsubata, T. (1998) Cutting edge: The B cell surface protein CD72 recruits the tyrosine phosphatase SHP-1 upon tyrosine phosphorylation, *J. Immunol. 160*, 4662−4665.
65. Daigle, I., Yousefi, S., Colonna, M., Green, D. R., and Simon, H.-U. (2002) Death receptors bind SHP-1 and block cytokine-induced anti-apoptotic signaling in neutrophils, *Nat. Med. 8*, 61−67.
66. Keilhack, H., Tenev, T., Nyakatura, E., Godovac-Zimmermann, J., Nielsen, L., Seedorf, K., and Bohmer, F. D. (1998) Phosphotyrosine 1173 mediates binding of the protein-tyrosine phosphatase SHP-1 to the epidermal growth factor receptor and attenuation of receptor signaling, *J. Biol. Chem. 273*, 24839−24846.
67. Klingmuller, U., Lorenz, U., Cantley, L. C., Neel, B. G., and Lodish, H. F. (1993) Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals, *Cell 80*, 1445−1454.
68. Tauchi, T., Damen, J. E., Toyama, K., Feng, G.-S., Broxmeyer, H. E., and Kryatal, G. (1996) Tyrosine 425 within the activated erythropoietin receptor binds Syp, reduces the erythropoietin required for Syp tyrosine phosphorylation, and promotes mitogenesis, *Blood 87*, 4495−4501.
69. Mason, J. M., Beattie, B. K., Liu, Q., Dumont, D. J., and Barber, D. L. (2000) The SH2 inositol 5-phosphatase Ship1 is recruited in an SH2-depedent manner to the erythropoietin receptor, *J. Biol. Chem. 275*, 4398−4406.
70. Bjorbaek, C., Lavery, H. J., Bates, S. H., Olson, R. K., Davis, S. M., Flier, J. S., and Myers, M. G., Jr. (2000) SOCS3 mediates

feedback inhibition of the leptin receptor via Tyr-985, *J. Biol. Chem. 275*, 40649−40657.
71. Okazaki, T., Maeda, A., Nishimura, H., Kurosaki, T., and Honjo, T. (2001) PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine, *Proc. Natl. Acad. Sci. U.S.A. 98*, 13866−13871.
72. Sheppard, K.-A., Fitz, L. J., Lee, J. M., Benarder, C., George, J. A., Wooters, J., Qiu, Y., Jussif, J. M., Carter, L. L., Wood, C. R., and Chaudhary, D. (2004) PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3ζ signalosome and downstream signaling to PKC*θ*, *FEBS Lett. 574*, 37−41.
73. Baznet, C. E., Gelderloos, J. A., and Kazlauskas, A. (1996) Phosphorylation of tyrosine 720 in the platelet-derived growth factor α receptor is required for binding of Grb2 and SHP-2 but not for activation of Ras or cell proliferation, *Mol. Cell. Biol. 16*, 6926−6936.
74. Cunnick, J. M., Mei, L., Doupnik, C. A., and Wu, J. (2001) Phosphotyrosines 627 and 659 of Gab1 constitutes a bisphosphoryl tyrosine-based activation motif (BTAM) conferring binding and activation of SHP-2, *J. Biol. Chem. 276*, 24380−24387.
75. Yu, W.-M., Hawley, T. S., Hawley, R. G., and Qu, C.-K. (2002) Role of the docking protein Gab2 in *β*1-integrin signaling pathway-mediated hematopoietic cell adhesion and migration, *Blood 99*, 2351−2359.
76. Wolf, I., Jenkins, B. J., Liu, Y., Seiffert, M., Custodio, J. M., Young, P., and Rohrshneider, L. R. (2002) Gab3, a new DOS/Gab family member, facilitates macrophage differentiation, *Mol. Cell. Biol. 22*, 231−244.
77. Kozlowski, M., Larose, L., Lee, F., Le, D. M., Rottapel, R., and Siminovitch, K. A. (1998) SHP-1 binds and negatively modulates the c-Kit receptor by interaction with tyrosine 569 in the c-Kit juxtamembrane domain, *Mol. Cell. Biol. 18*, 2089−2099.
78. Guntermann, C., and Alexander, D. R. (2002) CTLA-4 suppresses proximal TCR signaling in resting human CD4+ T cells by inhibiting ZAP-70 Tyr-319 phosphorylation: A potential role for tyrosine phosphatases, *J. Immunol. 168*, 4420−4429.
79. Hu, Y., Szente, B., Kiely, J.-M., and Gimbrone, M. A., Jr. (2001) Molecular events in transmembrane signaling via E-selectin, *J. Biol. Chem. 276*, 48549−48553.
80. Ali, S., and Ali, S. (2000) Recruitment of the protein-tyrosine phosphatase SHP-2 to the C-terminal tyrosine of the prolactin receptor and to the adaptor protein Gab2, *J. Biol. Chem. 275*, 39073−39080.
81. Gunaje, J. J., and Bhat, G. J. (2001) Involvement of tyrosine phosphatase PTP1D in the inhibition of interleukine-6-induced Stat3 signaling by α-thrombin, *Biochem. Biophys. Res. Commun. 288*, 252−257.
82. Kiener, P. A., Lioubin, M. N., Rohrschneider, L. R., Ledbetter, J. A., Nadler, S. G., and Diegel, M. L. (1997) Co-ligation of the antigen and Fc receptors gives rise to the selective modulation of intracellular signaling in B cells, *J. Biol. Chem. 272*, 3838−3844.

BI051408H