Probing Protein-tyrosine Phosphatase Substrate Specificity Using a Phosphotyrosine-containing Phage Library*

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Protein tyrosine phosphatases (PTPs) play important, highly dynamic roles in signaling. Currently about 90 different PTP genes have been described. The enzymes are highly regulated at all levels of expression, and it is becoming increasingly clear that substrate specificity of the PTP catalytic domains contributes considerably to PTP functionality. To investigate PTP substrate selectivity, we have set up a procedure to generate phage libraries that presents randomized, phosphotyrosine-containing peptides. Phages that expressed suitable substrates were selected by immobilized, substrate-trapping GST-PTP fusion proteins. After multiple rounds of selection, positive clones were confirmed by SPOT analysis, dephosphorylation by wild-type enzyme, and K_d determinations. We have identified distinct consensus substrate motifs for PTP1B, Sap-1, PTP-β, SHP1, and SHP2. Our results confirm substrate specificity for individual PTPs at the peptide level. Such consensus sequences may provide a tool for identifying potential PTP substrates and for the development of peptidomimetic inhibitors.

Protein-tyrosine phosphatases (PTPs) are members of a family of enzymes that control the phosphorylation state of proteins and thus play a major role in signaling. The dynamic character of intracellular phosphorylation and dephosphorylation reactions is best appreciated when cells are treated with generic phosphatase inhibitors, such as vanadate or arsenate, which result in the rapid and massive phosphorylation of multiple intracellular targets and which has pleiotropic physiological effects (1). Most PTPs show a lack of substrate selectivity when tested in vitro or when overexpressed in cells; therefore, establishing the physiological role of PTPs is difficult. Around 90 PTPs have been described (2, 3), including the dual-specificity phosphatases (those that also dephosphorylate serine-phosphate or threonine-phosphate).

Blocking individual PTPs may enhance specific signaling pathways (4, 5), but only a few PTPs have been linked convincingly to physiological substrates (4). However, the discovery that PTP1B is a negative modulator of insulin and leptin signaling has spurred considerable interest in this enzyme as a drug target (6–8). PTP1B has been shown to negatively regulate the insulin receptor tyrosine kinase (9–12). Flint et al. (13) have developed a strategy that uses catalytically inactive PTP “trapping” mutants that are still able to interact with their substrates but are unable to dissociate. Using these mutants, the Flint team and others (14, 15) have isolated and identified substrates from cells that had been treated with vanadate. By using a large panel of substrate-trapping PTP mutants, we have shown that PTP1B has unique substrate specificity for the phosphorylated insulin receptor (16). Thus, substrate specificity is important and useful to predict PTP function, and substrate-trapping PTP mutants are useful tools to study substrate specificity.

To define PTP substrate specificity in more detail, phosphopeptides have been used. Assays on random peptide libraries (17) or on chemicals (18) that mimic the recognition site of the pharmacological target of PTP1B have demonstrated a preference for acidic residues at positions −3 and −2 from the phosphorylated tyrosine and an aromatic group at position −1. More recently, a reverse alanine scan was performed to test the affinity of PTP1B for different short peptide sequences (19), and Asante-Appiah et al. (20) have tested T cell-PTP on a library of synthetic peptides, changing positions one by one with all amino acids except cysteine. These methods have resulted in “optimal” PTP phosphopeptide substrate motifs. On the basis of such information a selective inhibitor for PTP1B has been designed (21). More recently, we have used the “SPOT” assay, a high throughput, on-membrane phosphopeptide synthesis procedure to systematically investigate the optimal −1/+1 amino acid context of a phosphotyrosine for recognition by PTP1B (22).

To analyze PTP substrate specificity while starting from a large pool of potential substrates, we have used an M13 bacteriophage library, wherein each phage presents a unique phosphopeptide. The presented peptide is embedded in the rigid protein structure of a structural coat protein. The phage display technology has previously been used in studies of kinase selectivity (23–25). The use of a randomly phosphorylated library was also used to identify a consensus sequence for the PTB domain of Shc (24). More recently, a cDNA library for proteins that are expressed on phages has been used to identify ligands of the SH2 domain of SHP2 (26). For our study, we have chosen to display the library of peptides on the major capsid protein VIII of phage M13, which results in high copy number presentation (27). From earlier studies, it is known that extended incubation of this type of phage to Src-family kinases
results in extensive, non-selective tyrosine phosphorylation (24), an observation that we have exploited to study PTP substrate specificity.

In the present study, we have used immobilized, trapping PTP mutants to isolate phosphopeptide-presenting M13 phages to analyze PTP substrate specificity. The approach was tested first on PTP1B, for which consensus sequences have been identified (19). The sequences that were obtained were confirmed by using the SPOT assay (22). This latter approach, which involves the synthesis of phosphopeptides on membranes, enabled us to further modify individual amino acids in the sequences that were discovered. Following this analysis of PTP1B, we have performed new rounds of phage selection with PTP-Sap1 and PTP-β, which are closely related phosphatases that differ from PTP1B, and we demonstrated that they show distinct substrate preferences. However, by replacing one specific PTP-Sap1 amino acid, we were able to change the type of substrate that was selected in the mutated PTP. Finally, substrate specificities of the catalytic domains of PTP-SH2 and SHP2 are determined.

Taken together, our data confirm the ability of the PTP catalytic domain to select a preferred substrate in a complex population of displayed tyrosine-phosphorylated motifs, and they demonstrate the validity of this novel approach to study PTPs.

MATERIALS AND METHODS

Plasmid Construction and Protein Purification—PTPs were cloned and purified as described previously (16). Briefly, specific primers corresponding to the beginning and the end of the catalytic domain of every PTP was used to amplify by PCR an expressed sequence tag containing the region of interest. These primers were designed with an EcoRI or Muni site at the 5’ end and a NotI site at the 3’ end. These two restriction sites were used to clone the catalytic domain in frame into an EcoRI-NotI-cut PEX2TK vector (Amersham Biosciences). For the construction of the trapping mutants, we designed internal primers that create a D → A mutation, as described (16). Mutation for the Arg88 mutation, as described (16). Mutation for the Arg88 mutation, as described (16).

For the experiments described herein, an increase in the number of trapped clones was observed (bound and unbound ratio, a factor between 10^3–10^4). Furthermore this increase in the number of clones was phosphoryrosine-dependent: when the selected pool of phages was not phosphorylated prior to the trapping, no residual binding was observed (data not shown). Phagotices that purified as single colonies, and colony-PAR was performed by using a specific phage gene VIII primers: forward, 5'-ATT GTA GGC GCT CTT GCC GT-3' and reverse, 5'-AGC CCA AGA ACG CCA ATG TTC CTC GCO CTT ACG ACT G-3'. The clones used for SPOT analysis were subcloned into modified pEX2TK vector that encodes a protein kinase A phosphorylation site in-frame with GST. Constructs were controlled by sequencing. Escherichia coli BL21 (codon +; Stratagene) were transformed, and a single colony was grown in 25 mL of LB agar containing 50 mg/mL ampicillin and chloramphenicol at 37°C until an OD of 0.5 was reached. Protein production was performed at 30°C for 3 h after the addition of IPTG at a final concentration of 250 μM. The bacteria were pelleted and resuspended in lysis buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 0.1% Triton X-100, 150 mM NaCl) plus a proteinase inhibitor mixture, Complete™ (Roche Applied Science) and lysed by a treatment with lysozyme (200 μg/mL final) for 1 h on ice followed by three rounds of sonication. Supernatant of the lysate was incubated for more than 2 h with 100 μM of a 50% solution of glutathione-Sepharose beads (Amerham Biosciences) at 4°C. Finally, beads were extensively washed in PBST (PBS, 0.1% Triton X-100), and the PTPs were eluted in 50 mM Tris, pH 8.0, with 10 mM glutathione. Glycerol was added to a final concentration of 20%; the amount of proteins produced was determined using a Bio-Rad protein assay, and aliquots were stored at −20°C until use.

GST-PEP constructs were prepared as follows: the primers used correspond to the pVIII capsid sequence of M13 plus two restriction sites (XhoI and NotI): 5′-TAT GTC GAG TCT TTC GTC GAT GA-3′ for the sense and 5′-ATA GGG GCC GCT TGG AAG GAG TCA AAG GCC G-3′ for the antisense. The DNA of the plasmid was amplified directly by adding 10 phages to the PCR mix. After PCR using Hercu-lase polymerase (Stratagene), the DNA fragments (100 bp) were purified by using Microcon® PCR (Millipore); gel extraction was performed with Ultrafree®-DA (Millipore). The cloning and protein purification were finally accomplished following the same protocol as for the PTPs, with 0.5 only difference being that TB1 (Stratagene) bacteria were transformed, and a single colony was grown and induced for protein production and phosphorylation exactly as described by the manufacturer. Again, all constructs were checked by DNA sequencing before protein production.

Phosphorylation of the Phage Library and Enrichment—M13 phages (10^11 plaque-forming units) were incubated for 3 h at 30°C in kinase buffer (25 mM Tris, pH 7.5, 5 mM MgCl₂, 2.5 mM MnCl₂, 2.5 mM DTT, and 1 mM ATP) in total volume of 20 μL with 3 units of Src kinase for 3 h at 30°C.

The library was enriched through an anti-PTyr column (Upstate Biotechnology). In this experiment, we used the slurry in batch. About 40 μl of the column were incubated with the phosphorylation mix at 4°C overnight. The bound phages were eluted with phenylphosphate. These clones were amplified into TG1 cells and were passed twice again in the column. The amplified tyrosine-carrying phages were then stocked at 4°C in TE buffer and used for the trapping experiment (see below).

Panning, Amplification, and Sequencing of the Selected Phages—Phages (10^10) were incubated in kinase buffer (20 mM Tris, pH 7.5, 5 mM MgCl₂, 2.5 mM MnCl₂, 1 mM ATP, 2.5 mM DTT, and with or without 3 units of Src kinase) for 3 h at 30°C. (The supplier definition of one unit is the activity that is able to transfer 1 pmol of phosphate to cd2 peptide per min per ng.) The phage pool, purified from baculovirus, was purchased from Upstate Biotechnology. Glutathione-Sepharose beads were pre-coated with 3 μg of either GST or PTP-GST for 4 h at 4°C in a solution of Trapping buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1 mM DTT) with 1% bovine serum albumin final. The phages were first pre-cleared by using GST-beads for 30 min at 4°C and spun down; the supernatant was incubated with the coated PTP-GST for 4°C with a constant shaking. Finally, beads were spun down and washed several times (5x for the first panning, then 10x) with a solution of phosphate-buffered saline with 0.25% Tween final. Phages were eluted from PTP-GST by an acidic treatment (glycine buffer, pH 2.7) for 10 min at room temperature with a constant shaking, beads were spun down, and phages were recovered in the supernatant. One tenth volume of a 1x Tris, pH 9.0 solution was added to restore the pH. Phages were finally titered in both fractions (bound and unbound), and the rest of the bound fraction was used to infect XL1 MRF bacteria (Stratagene) following described procedures (27).

The day after, cells were sampled, and phages were amplified by using helper phage M13K07 (Amerham Biosciences) following the manufacturer's protocol in the frame R1 competent E. coli strain.

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Phosphorylation Assay on GST-PEP Constructs and on pNPP—100 ng of GST-PEP construct was incubated with 10 ng of PTP wild type in PTP buffer (50 mM Hepes, pH 7.4, 0.05% Nonidet P-40, and 1 mM DTT). The reaction was stopped by mixing equal volume aliquots with a solution of 50 mM activated sodium orthovanadate (Sigma) at different time points. The total mix was finally spotted on a nitrocellulose membrane using a 96-well dot blot apparatus (Bio-Rad) and the phosphorylation state of the substrate was visualized by using anti-phosphotyrosine antibody (clone G410, Upstate Biotechnology).

For pNPP hydrolysis, 200 ng of PTP were incubated with varying amounts of the generic substrate (0–600 nM) in assay buffer (50 mM Tris, pH 6.8, and 2 mM DTT) in a total volume of 50 μl at room temperature. Plates were read at 405 nm, and kinetic parameters were determined by using a non-linear regression software (Prism, GraphPad, San Diego).

SPOT Synthesis and Probing—Peptides were manually synthesized on derivatized cellulose membrane provided by Sigma-Genosys, which also provided the 20 Fmoc-amino acids active esters. Fmoc-phospho-tyrosine-dependent reactions were performed by coupling the reagent, N′-diisopropylcarbodiimide (Sigma) and hydroxylbenzoate (HOBT, Fluka; Ref. 28).

Labeling of GST-PTP for the SPOT analysis was performed as follows: 2–5 μg of GST fusion proteins were bound to glutathione-Sepharose beads (Amerham Biosciences) at 4°C. After washing, GST fusion proteins were eluted from beads in a solution of 50 mM Tris, pH 8.0, with 10 mM glutathione (28).

312 Probing PTP Substrate Specificity
Membranes were blocked (for at least 2 h) and probed at 4 °C in Western wash buffer (10 mM Tris, pH 7.4, 0.1% Triton X-100, and 150 mM NaCl) + SPOT blocking buffer (Sigma-Genosys). After 2 h, membranes were washed several times in Western wash buffer and were autoradiographed.

Enzyme Assays Using Phosphotyrosine Peptide Substrates—The dephosphorylation of phosphotyrosine-containing peptides catalyzed by PTPs was performed by using the continuous fluorometric assay described (29) using a PerkinElmer LS55 fluorometer. The reaction was started by the addition of enzyme, and the initial reaction rates at various peptide concentrations were measured ensuring less than 5% product formation.

### Results

**Preparation of a Phosphotyrosine-presenting Phage Library**—We have used an M13 library that presents random peptides of nine amino acids whose construction and use have been described earlier (24). This library was incubated with excess Src kinase, which results in relatively nonspecific library phosphorylation (24). Pilot studies using [γ-32P]ATP followed by autoradiography confirmed that phosphorylation occurred in the capsid protein of the library but not of wild-type M13, indicating that only tyrosines in the variable peptide had been phosphorylated (data not shown). The library was subsequently enriched for peptide-expressing phages that that could be phosphorylated by Src by purification on a column containing an immobilized antibody against phosphorysotyrine. Eluted phages were re-amplified in bacteria and phosphorylated (see “Materials and Methods,” and Fig. 1). Sequence analysis of a random subset of phages from this enriched library indicated that 13 of 14 phages encoded a peptide with one or more tyrosines, with little tyrosine context bias, except for a 30% occurrence of G at +1 and 23% P at −1 (Table I). The probability that one random nine-mer phage peptide contains one or more tyrosine residues is 25%/1 – (62/64)9; the probability that a set of 14 random peptides produces fewer than two peptides that lack tyrosines is only 0.02%((0.2514 × 0.75 × 14) + 0.7514). Therefore, we can conclude that the phage library is tyrosine-phosphorylated and enriched.

**Phage Display on PTP1B**—To assess the phage display procedure with trapping-PTP mutants (13), we have decided to start with PTP1B, whose substrate preference has been extensively investigated in several ways: (i) a number of potential PTP1B cellular substrates has been described (9, 10); (ii) a reverse alanine scan has been performed (19) which produced an optimal substrate consensus sequence; and (iii) the co-crystal structure of PTP1B with substrate peptides has been elucidated (30, 31).

After three rounds of selection of our tyrosine-enriched phage library with the PTP1B trapping mutant, an increase in the ratio of binding/flow-through phages was observed. DNA from phages that had been retained on the column was sequenced (Table II). In all cases, the sequenced peptide contained a tyrosine. Two sequences were found on multiple occasions (4-fold and 2-fold and encoded by the same DNA phages) was performed (19) which produced an optimal substrate consensus sequence; and (ii) the co-crystal structure of PTP1B with substrate peptides has been elucidated (30, 31).

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When a phage peptide contained multiple tyrosines, it was not clear at this point which of these had been phosphorylated by Src. To address this issue, we used the SPOT assay (22, 28). This technology involves the synthesis of (phospho)peptides on a solid support, followed by phosphorylation of the peptide on the solid support. The phosphorylated peptide can then be used to probe a membrane with immobilized antibodies. The ratio of bound to unbound phages can then be measured, allowing for the determination of the substrate specificity of the PTP. Table I shows a binding assay of PTP1B to the set of (phospho)peptides presented by the phage library.

### Table I

**Peptides presented by randomly selected phages**

| Phage | Sequence displayed |
|-------|--------------------|
| Ctrl-1 | EELLSEPRDEADPAK     |
| Ctrl-2 | EFEOYQGVNADPAK     |
| Ctrl-3 | EFQEVQOLSAPDAK     |
| Ctrl-4 | EFYVLSVGFEDPAK     |
| Ctrl-5 | EFARTYDAAPMDPAK     |
| Ctrl-6 | EFQHTYTVDPDPAK     |
| Ctrl-7 | EFHNYNISPADPAK     |
| Ctrl-8 | EFQGBELLTDPAK       |
| Ctrl-9 | EFYGSISPVASDPAK     |
| Ctrl-10 | EFCAVEINTLDPAK    |
| Ctrl-11 | EFAYTLTGSMDPAK     |
| Ctrl-12 | EFAQTYGFSNPDKA     |
| Ctrl-13 | EFVAQPFLWRSPDPAK   |
| Ctrl-14 | EFYGVSSLDPK        |

*Fig. 1. Strategy overview for the synthesis of the phosphorylated phage library and selection for phages with substrate specificity for a PTP. Src*, activated Src kinase.*
preference was displayed for PTP1B binding. Thus, in peptide 1B-1, phosphorylation of the first tyrosine (spot 2) resulted in much stronger binding than the second (spot 3). Peptide 1B-4, which has three tyrosines, shows that the second tyrosine was clearly implicated. In no case did double or triple phosphorylation produce stronger binding, suggesting that all phages were mono-phosphorylated. Nevertheless, spot 15 (clone 1B-4), which has two successively phosphorylated tyrosines, showed good binding. This is a well known motif that was reported earlier (31). Two peptides (1B-6 and -11) showed weak binding only. The results were used to annotate the phosphotyrosines (Z) at the correct positions in Table II.

In the alignment shown in Table II, in 6 of 14 sequences the tyrosine is preceded by amino acids EF (underlined), which are part of the phage capsid (which is encoded by one of the cloning restriction sites). This bias was also seen, but to lesser extent, in the “random group” (Table I) and may reflect an Src-preference. The PTP1B set has a very strong preference for a phenolic amino acid at position −1 (92%), which is not found in the random set (29%). It has been demonstrated that a phenolic group in −1 of the substrate tyrosine would stabilize the PTP1B-substrate interaction and orient the phosphate group in the catalytic pocket (30).

To further interpret our results, we tested peptide 1B-4, which produced the strongest binding (spot 11 in Fig. 2A) to a valine scan (Fig. 2B; assay as in Fig. 2A). This scan confirms the consensus recognition sequence for PTP1B (Table II, bottom) with critical positions at −1, +1, and +3. Interestingly, the +2 position allows for multiple amino acids.

Next, we tested whether sequences which had been selected by the trapping mutant could also be dephosphorylated by the wild-type PTP1B enzyme. The phage nucleotide sequence that encoded for the displayed peptide 1B-1 was sub-cloned into a GST expression vector (see “Materials and Methods”). The fusion protein was co-expressed with Elk tyrosine kinase in bac
alicia, purified, and incubated with wild-type PTP1B enzyme for dephosphorylation. We have observed previously that the GST tag is not phosphorylated by Elk kinase in TKB1 cells (data not shown). The fusion protein was efficiently dephosphorylated by PTP1B as measured by incubation with an anti-phosphotyrosine antibody (Fig. 2C). Finally, we have synthesized (soluble) phosphopeptides containing the sequences AEGFZATYG (1B-1) and AEGEFZGTYRG (1B-7). PTP1B was able to dephosphorylate these peptides with a $K_m$ of 11 μM. This value is comparable with the $K_m$ for previously identified optimal (19) or natural peptides (31).

Our results define the minimum PTP1B required sequence around the phosphotyrosine (Z) as EFZ/G/A(T/Y/G/A), with a preference for a nucleophilic amino acid at −2 (Ser or Thr), and at +3, a Tyr rather than a Phe (e.g. in 1B-13, the signal is weaker even if the rest of the consensus is conserved). Because this consensus corresponds very well to earlier published observations (17, 19, 22), we conclude that the phage display method that we have developed accurately yields PTP recognition motifs. However, it is not known at present if substrate specificities of trapping mutant and catalytically active wild-
type enzymes are identical for all PTPs.

**Phage Display on PTP-Sap1 and PTP-β—**PTP-Sap1 and PTP-β were chosen next because they differ from PTP1B but belong to the same subfamily of PTPs (2, 3). We wished to test the hypothesis that two related PTPs with a high sequence similarity can recognize different substrates. An enrichment in the number of phages was observed for both PTPs after three rounds (data not shown) and single clones were sequenced. PTP-Sap1 was very efficient in trapping; many positive clones could be detected easily, but PTP-β was able to select only three phages using this method, even though the wild-type version of this enzyme was highly active on artificial substrates (data not shown). The sequences obtained by Sap-1 selection were reminiscent but not identical to those obtained for PTP1B (Table III). An amino acid with an aromatic group at position 1 and 3 (with a preference for Phe) and an invariant glycine at position 1 seems to be the minimum consensus sequence that is recognized by Sap-1. A very strong preference was seen for the EF motif preceding the phospho-Tyr. In the only peptide where the EF motif was not used (Sap-42), another acidic-hydrophobic amino acid combination emerged (DV). The three clones that were selected by PTP-β showed more variation. In all three peptides, the EF sequence was separated from the phosphotyrosine by an additional, uncharged amino acid, and there was no preference seen for a glycine at the +1 position.

Again, several peptides were expressed as a GST-fusion, phosphorylated, and incubated with wild-type Sap-1 or PTP-β. As shown in Fig. 3, both phosphatases efficiently dephosphorylated their corresponding phosphopeptides.

Finally, synthetic peptides that were predicted as substrates for PTP-Sap1 were designed, synthesized, and tested with Sap1 (Table III). Both sequences that were selected were good Sap-1 substrates, with $K_m = 7$ and 10 μM.

**SPOT Analysis of the Positive Sequences for PTP-Sap1—**As for PTP-1B, we tested the positive clones that were found for PTP-Sap1 (Table III) by SPOT. The results of this analysis (Fig. 4A) were used again to annotate the phosphotyrosines (Z) at the right positions in Table III. In every case where the aromatic amino acid was a tyrosine (rather than a phenylalanine), phosphorylation resulted in reduced binding (peptides Sap-2, -3, and -5).

Next, a valine-scan was performed on Sap-5 because this sequence was found twice (Fig. 4B). Spots 9–12 confirm the critical requirement of an acidic amino acid at position −2, the phenylalanine at −1, and a glycine at +1; however, the valine is tolerated as a replacement of tyrosine at +3. These require-
To test this hypothesis, we have created a PTP-Sap1R88N mutant in both a wild-type and a trapping-PTP mutant background. We first confirmed that the catalytic activity of the PTP-Sap1R88N single mutant could still convert a generic phosphatase substrate (pNPP) with a $K_{\text{m}}$ that was indistinguishable from the wild-type enzyme (Table IV).

We then used the trapping/R88N double mutant to select phosphopeptide-presenting phages. After three rounds of selection, we observed an enrichment of retained phages (data not shown), and DNA from a number of phages was sequenced. The sequences that were obtained were similar to those obtained from the (single) trapping mutant Sap-1 (Table V), which is characterized by a Gly at +1 and an aromatic Phe/Tyr at +3 (with a tolerance for other hydrophobic amino acids such as Ile or Val). However, in the majority of cases (75%), the EF capsid sequence was then separated from the phosphotyrosine by a Leu, Thr, or Ile, precisely as for the phages that had been selected by PTP-β (Table III).

Again, the sequences obtained were tested on SPOT. In this case, the peptides were tested against both the Sap-1 double R88N/DA and single DA trapping mutant. As shown in Fig. 6, binding by the wild-type version of Sap-1 (Panel B) in most cases was much weaker than for the R88N mutant. The only exception is peptide Sap*-11 (spots 13–14), which has an atypical EFZ motif.

Thus, PTP-Sap1 R88N has become “PTP-β-like” in its substrate preference, suggesting that the amino acid that corresponds to Arg-47 in PTP1B plays a major role in substrate recognition in other phosphatases as well, and that our approach allows picking up subtle PTP catalytic domain preferences.

**Consensus Substrate Sequences for SHP1 and SHP2**—Finally, the catalytic domains of SHP1 and SHP2 were subjected to the same phage-display analyses. SHP1 and -2 are another subgroup of phosphatases, both of which have SH2 domains (not included in our GST constructs), and whose catalytic domain ancestry can be traced to *Caenorhabditis elegans* and *Drosophila* (33). As our results show (Table VI), both phosphatases tend to prefer an alanine at position +1, although this preference is much stronger for SHP1. As for other phosphatases, the position at +2 allows for variation, whereas a hydrophobic amino acid is preferred at +3. Remarkably, SHP2 has a strong preference for a basic amino acid at position +5 (86% versus 28% for SHP1). Again, these findings are supported by earlier observations (34).

**DISCUSSION**

We show here for the first time how phage libraries can be used to define phosphatase substrate specificity. Key to our approach was phosphorylation of the library using saturating amounts of Src (24), resulting in little or no bias of the context in which the phosphotyrosines were presented by the phage. Second, the library was enriched, prior to use, for phages that have shown that the catalytic activity of the PTP-Sap1R88N single mutant could still convert a generic phosphatase substrate (pNPP) with a $K_{\text{m}}$ that was indistinguishable from the wild-type enzyme (Table IV).

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**Modification of the PTP-Sap1 Substrate Consensus by Mutating a Key Residue within the Sap-1 Catalytic Pocket**—The sequence similarity between the catalytic domains of PTP-β and PTP-Sap-1 is relatively high (>50%). Nevertheless, we had found that the number and the identity of positive phages differed for each PTP. We took advantage of the fact that the crystal structure of PTP1B with a substrate peptide had been resolved (30, 31); that structure shows an interaction between Arg-47 (catalytic domain numbering; Refs. 19, 20, 30–32) and Phe-1 in an optimal PTP1B peptide substrate. A simple alignment of their catalytic domains shows that the corresponding amino acid at this position for PTP-Sap1 is also an Arg (Arg-88), but in PTP-β we find an Asn (Asn-101) at this position (Fig. 5). We hypothesized that this conservation explains why both PTP1B and PTP-Sap prefer a Phe at position −1, but that PTP-β might not share this preference.

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Beyond this, the enzyme interacts with more than just the few amino acids that surround the phosphotyrosine substrate, as discussed previously (36, 37). On the one hand, interaction between PTP1B and the insulin receptor seem to involve a large linear stretch of the receptor (31). On the other hand, we have shown that PTP1B interacts with its insulin receptor substrate at multiple motifs, some of which are clearly not

**Fig. 4. PTP-Sap1 binds the EFZG motif on the SPOT-binding assay. A, PTP-Sap1 binding confirmation of peptides derived from the phage display. 15-mer peptides of the phages from Table III were synthesized on membrane, with and without phosphotyrosine(s) (Z). Binding of the radiolabeled PTP-Sap1DA was revealed by autoradiography. B, mapping, by SPOT, of the binding site of the clone Sap-5 by a valine scan. The membrane was again probed with the radiolabeled PTP-Sap1DA.
probed with the PTP-Sap1 DA (single) trapping mutant. B was revealed by autoradiography.

15-mer peptides of the phages Sap1R88N selected by PTP-Sap1 to sequences from phages that were selected by PTP-Sap1 R88N mutant on pNPP. Lastly, sequences that form an optimized motif around the Arg47 residue of PTP1B that interacts with the amino acid phenolic group in position 3, with an optimal peptide sequence, ELEFZMDYE. Finally, we present novel consensus sequences for PTP-Sap1, PTP-Sap1 R88N, and wild-type Sap1 to sequences from phages that were selected by PTP-Sap1 R88N. A, PTP-Sap1 R88N binding confirmation with peptides derived from the eight-phage clones. 15-mer peptides of the phages listed in Table V were synthesized on a membrane, with and without phosphotyrosine(s) (z). Binding of the radiolabeled PTP-Sap1 R88N was revealed by autoradiography. B, the same membrane was reprobed with the PTP-Sap1 DA (single) trapping mutant.

Different sets of phage sequences that are selected by the different PTPs nevertheless clearly indicate substrate preferences, which we have confirmed by resynthesis on membranes (22), valine scans, and K<sub>m</sub> determinations. Furthermore, we show that modifying a critical PTP amino acid residue can change its substrate specificity.

Our proof-of-concept phage selection that used PTP1B yielded a consensus that closely matches previous data that showed that modifying a critical PTP amino acid residue can change its substrate specificity. The consensus sequences that we have found are not absolute; as reported previously (30), there exists considerable flexibility for PTPs to recognize phosphopeptides that deviate from their "ideal" substrate. This concept is being used as a working model to design PTP-inhibitors (21, 38).

Finally, we have confirmed the relevance of a specific cata-

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**TABLE IV**

| Phage    | K<sub>m</sub> (mM) |
|----------|-------------------|
| Sap-1 WT | 2.67 ± 0.46       |
| Sap-1 R88N | 2.67 ± 0.23        |

**TABLE V**

PTP-Sap1 R88N mutant selected phage sequences

| Phage     | Sequence displayed | Number |
|-----------|--------------------|--------|
| Sap*-1    | EFHNLZGTPREDPAK    | 1      |
| Sap*-2    | EFQATGVTSPQPAK     | 2      |
| Sap*-7    | EFLGE IQTQPqPAK    | 3      |
| Sap*-8    | EFLZANVERSSPAK     | 4      |
| Sap*-10   | EFLZQIIPRSPPAK     | 5      |
| Sap*-11   | EFQCGDDHQPAK       | 6      |
| Sap*-12   | EFQGE YRPqQPAK     | (2x)   |
| Sap*-15   | EFGSFHQADPAK       | (5x)   |

Consensus

-2: 50% F, 25% E
-1: 100% hydrophobic
+1: 88% G
+2: X
+3: 100% hydrophobic

**TABLE VI**

SHP1 and SHP2 selected phage sequences

| Phage     | Sequence displayed   | Number |
|-----------|----------------------|--------|
| SHP1-1    | EFLAQIYRPPEPAK      | (2x)   |
| SHP1-4    | EFPYMATMGLDPAK      |        |
| SHP1-5    | EFLAYKVGSDPAK       |        |
| SHP1-6    | EFLAVGSSPPEPAK      |        |
| SHP1-7    | EFLMVNVNPPEPAK      |        |
| SHP1-8    | EFLAVGSSPPEPAK      | (2x)   |
| SHP1-10   | EFLAVGSSPPEPAK      |        |

Consensus

-2: 67% E, 22% P
-1: 86% hydrophobic (57% F)
+1: 86% A
+2: X
+3: 90% hydrophobic (56% V, 22% I)
lytic domain residue predicted to be directly involved in substrate recognition. Indeed, a single mutation was able to change the preferred substrate motif at a precise position. This suggests that a network of interactions between the catalytic core of the enzyme and its substrate could be the key to understanding substrate recognition. It may be very difficult to translate the motifs that we have identified into physiological cellular targets simply by sequence similarity and data base searching. But, in agreement with Vetter et al. (19), we do believe that our findings can help in the design of PTP inhibitors for new drugs, starting from peptide mimetics that are based on the catalytic domain, thus inhibiting the activity of a PTP for its natural substrate(s) (39).

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