The substrate specificity of the catalytic domain of SHP-1, an important regulator in the proliferation and development of hematopoietic cells, is critical for understanding the physiological functions of SHP-1. Here we report the crystal structures of the catalytic domain of SHP-1 complexed with two peptide substrates derived from SIRPα, a member of the signal-regulatory proteins. We show that the variable β5-loop-β6 motif confers SHP-1 substrate specificity at the P-4 and further N-terminal subpockets. We also observe a novel residue shift at P-2, the highly conserved subpocket in protein-tyrosine phosphatases. Our observations provide new insight into the substrate specificity of SHP-1.

Protein-tyrosine phosphatases (PTPs) consist of a diverse family of enzymes that play crucial roles in cell growth, differentiation, and transformation (1–3). They can be broadly divided into membrane-bound, receptor-like PTPs, and cytosolic PTPs. The cytosolic PTPs contain only one catalytic domain, whereas the membrane-bound receptor-like PTPs usually contain two tandem catalytic domains. The catalytic domains of PTPs are highly conserved in their three-dimensional structures (4–7). However, they have remarkably different substrate specificity (3, 8–10), which is still not well understood. Previous studies using various synthetic phosphotyrosyl peptides studied were not derived from physiological substrates (4–7). However, they have remarkably different substrate specificity (3, 8–10), which is still not well understood. Previous studies using various synthetic phosphotyrosyl peptides failed to identify a shared by PTP substrate because the peptides studied were not derived from physiological substrates of PTPs. In the present study, we have addressed the structural basis for the substrate specificity of PTPs using SHP-1 and its physiological substrate SIRPα/SHPS-1 as a model. SIRPα is a transmembrane protein of the signal-regulatory protein family. Its extracellular domain contains three immunoglobulin domains, and its cytoplasmic domain contains four phosphotyrosine sites (Tyr(P)427, Tyr(P)505, Tyr(P)509, and Tyr(P)650).

SHP-1 is expressed primarily in hematopoietic cells, and contains two Src homology 2 (SH2) domains, a neighboring catalytic domain, and a C-terminal tail. Its phosphatase activity is inhibited by both the SH2 domains and the C-terminal tail (11, 12). SHP-1 is activated upon the binding of its tandem SH2 domains to immunoreceptor tyrosine-based inhibitory motifs. Domain-swapping studies on SHP-1 and its analogue, SHP-2, have shown that the catalytic domains of SHP-1 and SHP-2 have distinct substrate specificity (9, 10), and therefore illustrate that the dissection of the structural basis for the substrate specificity of SHP-1 is fundamental to the understanding of its physiological functions. The identification of the substrates of SHP-1 (i.e. SIRPα, CD22, and CD72; Refs. 13–15) has made it possible for us to probe this structural basis. The results of this probe are presented below.

EXPERIMENTAL PROCEDURES

Crystallization and Data Collection—The C46SS mutant of the SHP-1 catalytic domain (245–532) was cloned, expressed, and purified as described elsewhere (16). The phosphotyrosyl decapeptides were synthesized and purified to 90% purity by SynPep Inc. (Dublin, CA). Crystals of the native enzyme were obtained by vapor diffusion method with 5 mg/ml protein drops equilibrating over reservoir solution containing 1.9 M (NH₄)₂SO₄, 0.1 M Tris-HCl, pH 8.5. The Tyr(P)469 complex crystals were obtained by co-crystallization method with the protein and the peptide at a 1:1 molar ratio. The Tyr(P)495 complex crystals were grown by soaking native crystals in cryosolvent (30% sucrose, 1.9 M (NH₄)₂SO₄, 0.1 M Tris-HCl, pH 8.5) containing 0.5 mM peptide Tyr(P)469 for 2–3 days. A data set of the Tyr(P)469 complex diffraction up to 2.5 Å was collected at −188 °C using an ADSC cryosystem and MAR imaging plate (Area Detector System Corp., Poway, CA); the x-ray source was generated from a Rigaku (Japan) RU-300 operating at 50 kV and 100 mA. A data set diffraction to 2.3 Å resolution for Tyr(P)495 complex was collected at −180 °C on F1 beamline at CHESS (Cornell University). Both data sets were processed with DENZO and SCALPACK (17). The crystal statistics are summarized in Table I.

Structure Determination—Crystal structures of both complexes were solved by molecular replacement method with program AMoRe (18), using coordinates of the native enzyme (Protein Data Bank code 1GWZ) as the search model. The molecular replacement solution for each complex structure was refined by rigid body and positional protocols in X-PLOR (19). The peptide positions were identified from the initial electron density difference map (Fo − Fc). However, the electron densities for the peptides were not quite continuous. After several cycles of model rebuilding and refinements, the electron densities for the peptides became clearer, and the peptides were added to the models. The complex models were then subjected to X-PLOR refinement, using the slow cool and positional refinement protocols with the 2 σ cutoff data between 6 Å and the highest resolution. The R-free value was also calculated from the beginning to the penultimate cycle by randomly selecting 10% data as the test set. The model building interspersed with X-PLOR refinement was done by the program TURBO-FRODO (20). In the penultimate cycle, the R-free values were 30.3% and 27.2% for the Tyr(P)469 and Tyr(P)495 complexes, respectively. The final crystallographic R-values for the Tyr(P)469 and Tyr(P)495 complexes were 19.9 and 20.5%, respectively, with all reflections used in the refinement. Fifty-three water molecules were added in the final Tyr(P)495 complex model; however, no water molecules were added in the final Tyr(P)469 complex model. The final refinement results and geometry analyses for each complex are also summarized in Table I.
Identification of in Vitro Substrates for SHP-1—SIRPα has been identified as a potential physiological substrate of SHP-1 in macrophages (13). However, the dephosphorylation sites of SHP-1 on SIRPα are still unknown. To identify these sites, we synthesized four phosphotyrosyl decapeptides corresponding to the four phosphotyrosine sites in the cytoplasmic domain of SHP-1 (defined in Fig. 2). With respect to amino acid sequence, the four synthetic peptides fall into two groups: Tyr(P)427 and Tyr(P)469, and Tyr(P)452 and Tyr(P)495. The two peptides in each group have very similar sequences for the N-terminal side of the Tyr(P) subpocket. It was formed by hydrocarbons of residue Pro P-5, Pro P-5 swept away from the Tyr(P) subpocket and interacted with the phosphate group i.e. SerP-2 of peptide Tyr(P) 495 into the P-1 subpocket, which was bound to the same binding subpockets of SHP-1 with similar orientations and conformations, except for a slight difference at the P+4 subpocket. The C-terminal residues of the peptides interacted mainly with loop αl/β1 and the α5-loop-α6 motif of SHP-1 (defined in Fig. 2).

The P-2 Subpocket—The P-2 subpocket was the most distinct subpocket at the N-terminal side of the Tyr(P) subpocket. It was formed by hydrocarbons from the side chains of Lys466 and Arg477 and by side chain of Tyr478. In the Tyr(P)469 complex, the P-2 subpocket was occupied by Leuα1-2 (Fig. 1, C and E). Surprisingly, it was occupied by PheP-3 in the Tyr(P)495 complex structure, indicating a likely residue shift at the N-terminal side of residue Tyr(P) in the Tyr(P)469 complex relative to the Tyr(P)495 complex. Because of this novel residue shift, the conformations of residue Tyr(P) were also different between the two complex structures. By shifting into the P-2 subpocket, PheP-3 pushed Serα1-2 of peptide Tyr(P)495 into the P-1 subpocket, which was occupied by Thrα1-1 in the Tyr(P)469 complex structure. At the same time, it pushed Ginα1-1 of peptide Tyr(P)495 toward the solvent (Fig. 1D).

The Subpockets N-terminal to the P-2 Subpocket—The kinetic studies showed that the substrate specificity of SHP-1 catalytic domain was determined mainly by residues N-terminal to residue Tyr(P) in the peptides. Between the Tyr(P)469 and Tyr(P)495 complexes, significant conformational differences were observed for peptide residues N-terminal to the P-2 subpocket (Fig. 1E). These N-terminal residues interacted mainly with the β5-loop-β6 motif of SHP-1. Therefore, the β5-loop-β6 motif determines, at least in part, the substrate specificity of SHP-1. In both complex structures, the β5-loop-β6 motif moved approximately 6.5 Å toward the N termini of the peptides and formed different interactions with the substrates. In the Tyr(P)469 complex, AspP-4 formed a salt bridge with residue Arg460 of the β5-loop-β6 motif. This salt bridge was further stabilized by two hydrogen bonds between Arg460 and Asn461 (Fig. 2A). These suggest that either an aspartate or a glutamate will be preferred for the P-4 position of the peptide substrates of SHP-1. In the Tyr(P)495 complex structure, ProP-5 was the corresponding residue to AspP-4. Because of the repulsion between the guanidine group of residue Arg460 and the hydrocarbons of residue ProP-5, ProP-5 swept away from the SHP-1 molecule into the solvent. Residue Arg460 also underwent a conformational change and formed weak van der Waals’ interactions with residue ProP-5. Other N-terminal residues, such as Thrα1-3 and Ginα1-1 of peptide Tyr(P)469 and SerP-4 in peptide Tyr(P)495, were exposed to the solvent.

The Tyr(P)-binding Subpocket—Like other PTPs, the signature motif of SHP-1, i.e. HCXAXGXR/S/T, formed the base for the Tyr(P) subpocket and interacted with the phosphate group of residue Tyr(P) by extensive hydrogen bonds in both complex structures (Fig. 2). Because of different binding at the P-2

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**Table I**

| Crystal data and refinement results | Tyr(P)469 | Tyr(P)495 |
|-----------------------------------|----------|----------|
| Crystal data                      |          |          |
| Unit cell (Å)                     | a = 111.58, b = 45.21, c = 56.27 | a = 112.68, b = 45.80, c = 57.47 |
| Space group                       | P21212   | P21212   |
| Resolution (Å)                    | 2.5      | 2.3      |
| Number of measured reflections    | 27345    | 45031    |
| Number of unique reflections      | 8998     | 13736    |
| Data completeness (%)             | 85.0     | 99.3     |
| Rwork (%)                        | 19.9     | 20.5     |
| Rfree (%)                        | 30.3     | 27.2     |
| Resolution range (Å)              | 6–2.5    | 6–2.3    |
| Number of reflections used in refinement | 7199 | 10547 |
| Number of non-H protein atoms     | 2290     | 2249     |
| Number of non-H peptide atoms     | 85       | 83       |
| Number of water molecules         | NA*      | 53       |
| r.m.s. differences for bond distances (Å) | 0.007 | 0.006 |
| r.m.s. differences for bond angles (°) | 1.4 | 1.3 |
| Average B-factor for protein (Å²) | 28.1     | 34.5     |
| Average B-factor for peptide (Å²) | 50.6     | 75.4     |
| Average B-factor for water molecules (Å²) | NA | 38.9 |

*a* NA, not applicable.

**Table II**

| Peptides | K_m (mM) | k_cat (s⁻¹) | k_cat/K_m (s⁻¹ mM⁻¹) |
|----------|----------|-------------|----------------------|
| Tyr(P)469 (EDTLTydALLD) | 80.0 | 141.7 | 1767.5 |
| Tyr(P)495 (PFSFSePYSVQ) | 71.5 | 80.0 | 1119.5 |
| Tyr(P)477 (TNDFtydALLN) | 312.0 | 217.7 | 697.8 |
| Tyr(P)495 (NHdETHePyASIQ) | 286.0 | 268.1 | 940.4 |

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The kinetic parameters for the catalytic domains of SHP-1 against the four synthetic phosphotyrosyl peptides.
subpocket, the Tyr(P)\textsuperscript{469} and Tyr(P)\textsuperscript{495} complexes had different conformations for residue Tyr(P) at the active site and therefore had different hydrogen bond networks. The O\textsubscript{g} atom of Ser\textsuperscript{455} was 2.6 Å from the phosphorus atom of residue Tyr(P) in the Tyr(P)\textsuperscript{469} complex structure and 2.3 Å from the phosphorus atom of residue Tyr(P) in the Tyr(P)\textsuperscript{495} complex structure. The walls of the Tyr(P) subpocket were formed by residues Tyr\textsuperscript{278}, Ser\textsuperscript{456}, Ile\textsuperscript{459}, Lys\textsuperscript{362}, Gln\textsuperscript{502}, and Gln\textsuperscript{506}. The most prominent residue was Tyr\textsuperscript{278} in loop α1/β1, which formed a stable π–π interaction between its phenyl ring and the side chain of residue Tyr(P) in the Tyr(P)\textsuperscript{469} complex structure. However, this π–π interaction was much weaker in the Tyr(P)\textsuperscript{495} complex structure because of the different conformations of Tyr\textsuperscript{278} and Tyr(P). The results indicated that peptide Tyr(P)\textsuperscript{469} had stronger binding affinity and was a more favorable \textit{in vitro} substrate for SHP-1, which is consistent with the kinetic study results (Table II). In addition, Tyr\textsuperscript{278} of the enzyme was involved in defining the depth of the Tyr(P) subpocket, making the sub-

Fig. 1. A and B, the electron density maps (2F\textsubscript{o} – F\textsubscript{c}) for the phosphotyrosyl peptide sites in both Tyr(P)\textsuperscript{469} (A) and Tyr(P)\textsuperscript{495} (B) complexes. The maps are contoured at 1.0 σ to 2.5 Å (A) and 2.3 Å (B), respectively, with the refined models of the decapetides in yellow. The amino acids are labeled. C and D, ribbon representations of the Tyr(P)\textsuperscript{469} (C) and the Tyr(P)\textsuperscript{495} (D) complex structures. The peptides are shown in the stick model. The catalytic domain of SHP-1 is shown in green. Structures of the catalytic domain in the two complexes were almost identical, with an r.m.s deviation of 0.5 Å. They were also similar to the native catalytic domain structure (7), with an r.m.s deviation of 0.8 Å. This Fig. was prepared by SETOR (25). E, comparison of peptides Tyr(P)\textsuperscript{469} and Tyr(P)\textsuperscript{495} after superimposing two complexes on their catalytic domains. Peptides Tyr(P)\textsuperscript{469} and Tyr(P)\textsuperscript{495} are shown in yellow and blue, respectively.
Substrate Specificity of SHP-1—We have determined that the β5-loop-β6 motif and loop α1/β1 determined the substrate specificity of SHP-1. We also observed the novel residue shift at the P-2 subpocket. The P-2 subpocket can bind either the P-2 or the P-3 residue of phosphotyrosyl substrates, provided that they are hydrophobic residues. To our knowledge, this is the first time that a residue shift has been observed in protein-protein recognition. This novel residue shift suggests peptides containing the sequence (L/I/V)X_pYXX(L/I/V) (n = 1 or 2) be potential substrates for SHP-1. The formation of the salt bridge between residue Arg460 of SHP-1 and residue AspP-4 of peptide Tyr(P)(469) in the Tyr(P)(469) complex structure suggests that peptides having the consensus sequence (D/E)X(L/I/V)X(X)XX(L/I/V) (n = 1 or 2) are favored as substrates of SHP-1. This consensus sequence explains why peptides Tyr(P)(427) and Tyr(P)(452) are not in vitro substrates for SHP-1. Recently, signal transduction co-receptors CD22 and CD72 were identified as both the activators and substrates of SHP-1 (14, 15). Using the consensus sequence, we predict that sites Tyr777, Tyr837, and Tyr857 of CD22 and sites Tyr7 and Tyr39 of CD72 are the dephosphorylation sites of SHP-1.

The catalytic domain swapping studies on SHP-1 and SHP-2 have demonstrated the different substrate specificity of the catalytic domains of SHP-1 and SHP-2 (9, 10). Among the six identified substrate-binding subpockets other than the Tyr(P) subpocket, only the P-4 subpocket differs between SHP-1 and SHP-2 (Fig. 4). Residue 360, which is located at the β5-loop-β6 motif, is an arginine in SHP-1 and a lysine in SHP-2. This residue difference is one of the main reasons for the different substrate specificity of SHP-1 and SHP-2. In addition to residue 360, several other residues in the β5-loop-β6 motif also differ between SHP-1 and SHP-2. These residues confer specific recognition for P-4 and further N-terminal residues of the substrate.

Comparison with PTP1B-Hexapeptide Complex—Comparison of the Tyr(P)(469) and Tyr(P)(495) complex structures with the PTP1B-hexapeptide structure (21) (Fig. 3) indicates significant conformational differences for residues on the N-terminal side of residue Tyr(P) between the Tyr(P)(469) and Tyr(P)(495) complex structures and the PTP1B-hexapeptide structure. The N terminus of the hexapeptide was positioned away from the PTP1B enzyme molecule and extended into the solvent, whereas the N termini of peptides Tyr-P(469) and Tyr-P(495) bound much closer to the catalytic domain of SHP-1. Based on the PTP1B-hexapeptide structure, Arg47 of PTP1B (equivalent to Arg279 of SHP-1) was proposed to recognize and stabilize the hexapeptide through two hydrogen bonds (21). However, we propose that the different substrate specificity of PTPs is not determined by Arg47, because it is highly conserved among PTPs (Fig. 4). In contrast to PTP1B, which shows indiscriminate low Km and high kcat/Km toward phosphotyrosyl peptides (22, 23), SHP-1 exhibits much higher kcat/Km toward peptides Tyr(P)(469) and Tyr(P)(495) than other phosphotyrosyl peptides (24). The high Km values of SHP-1 toward peptide Tyr(P)(469) and Tyr(P)(495) are due to the fact that the substrate specificity of

**FIG. 2. Schematic representation of the hydrogen bonds formed between the catalytic domain of SHP-1 and peptides Tyr(P)(469) (A) and Tyr(P)(495) (B) in the Tyr(P)(469) and Tyr(P)(495) complex structures, respectively.** Residues labeled with asterisks are from symmetry-related molecules. In addition to the hydrogen bonds, peptides Tyr(P)(469) and Tyr(P)(495) also interacted with the catalytic domain of SHP-1 by van der Waals’ interactions. Besides the Tyr(P) subpocket, we also identified six well-defined substrate-binding subpockets (P-4, P-2, P-1, P+2, P+3, and P+4). The P-4 subpocket was formed mainly by residue Arg360 from the β5-loop-β6 motif. The P-2 subpocket was formed by residue Lys362 from the β5-loop-β6 motif and residues Arg377 and Tyr377 from loop α1/β1. The Tyr(P) subpocket was formed mainly by the PTP signature motif (HCXAGKGR(S/T)) and residue Tyr375 from loop α1/β1. The P+1 subpocket was formed by residues Tyr279, Asn280, and Ile281 from loop α1/β1. The P+2 subpocket was formed by residue Gln492 from the α5-loop-α6 motif. The P+3 subpocket was formed by residue Ser493 from the α5-loop-α6 motif, residue Ile281 from loop α1/β1, and residue Lys375. The P+4 subpocket was formed mainly by residues Gln491 and Arg494 from the α5-loop-α6 motif.
SHP-1 is conferred by both the catalytic domain and the two tandem SH2 domains. Subcellular relocation of SHP-1 by its SH2 domains would significantly increase the local substrate concentrations. Therefore, the SHP-1-peptide complex structures are better representations of the in vivo PTP-substrate interactions than the PTP1B-hexapeptide structure.

Comparison with Other PTPs—

Residues forming the six substrate-binding subpockets other than the Tyr(P) subpocket were distributed around loop α1/β1, the β5-loop-β6 motif, and the α5-loop-α6 motif. Because the catalytic domains of PTPs show a highly conserved three-dimensional structure, we aligned the amino acid sequences of 21 different PTPs around these three substrate-binding regions (Fig. 4). In addition to the hyper-variable β5-loop-β6 motif, the P-3 and P-4 subpocket-forming residues in the α5-loop-α6 motif were also variable in PTPs. This finding strongly suggests that P-3 and P-4 residues in the substrates also can be specific recognition sites for PTPs and that the recognition is conferred by the α5-loop-α6 motif of PTPs.

Residues forming the P-2 subpocket are highly conserved among PTPs, except for Yersinia PTP, suggesting that the observed residue shift at the P-2 subpocket is also present in other PTPs. Therefore, the P-2 subpocket contributes to the binding affinity of PTPs toward their substrates, without necessarily determining their substrate specificity. Residue 360, the P-4 subpocket-forming residue located in the hyper-variable β5-loop-β6 motif, is either an arginine or a lysine in most PTPs. Therefore, those PTPs will prefer either an aspartate or a glutamate residue at the P-4 position of the peptide substrates. Other residues within the β5-loop-β6 motif are highly variable among PTPs. These residues likely are involved in the formation of the P-4 subpocket and its interaction with further N-terminal residues of the peptide substrates. The above analyses indicate that PTP substrate specificity comes from two regions: the P-4 and further N-terminal sites and the P-3 and P-4 sites of the substrates. The recognition of these two regions was conferred by the β5-loop-β6 and α5-loop-α6 motifs, respectively.
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