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Substrate Specificity of Protein Tyrosine Phosphatase: Differential Behavior of SHP-1 and SHP-2 Towards Signal Regulation Protein SIRPα1

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Abstract The substrate specificity of catalytic domains and the activation of full length protein tyrosine phosphatases, SHP-1 and SHP-2 have been investigated using synthetic phosphotyrosyl peptides derived from SIPRα1. We found that the catalytic domains of SHP-1 and SHP-2 exhibit different substrate specificity towards a longer trideca-peptide pY^{469+3} ($^{-7}$RPEDTLTpYADLDM^{+5}) and not to the shorter decapeptide pY^{469} ($^{-5}$EDTLTpYADLD^{+4}), the former being the substrate of SHP-2 only. Furthermore, the activation of full-length SHP-1 and not the SHP-2 by the deca/trideca-peptides suggested SIRPα 1 to be possibly acting as both an upstream activator and a substrate for SHP-1, and merely as the downstream substrate for SHP-2 in signaling events. J. Cell. Biochem. 84: 840–846, 2002. © 2002 Wiley-Liss, Inc.

Key words: substrate specificity; SHP-1; SHP-2; catalytic domain; SIPRα1

SHP-1 and SHP-2 are highly homologous cytosolic protein tyrosine phosphatase (sharing 60% overall sequence identity) consisting of two tandem SH2 domains, followed by a catalytic domain (PTP domain), and a C-terminal tail. In spite of having highly homologous structures, they display a distinct tissue distributions and biological functions [Neel and Tonk, 1997]. SHP-1 is mostly restricted to hematopoietic and epithelial cells, whereas SHP-2 is expressed in almost all cell types. Both associate with the activated receptors under in vivo and in vitro conditions via their SH2 domains, which recognizes specific pTyr residues on these receptors [Lechleider et al., 1993; Sugimoto et al., 1994]. They perform opposing functions inside the cells. SHP-1 negatively regulates a large number of different signaling pathways including those associated with cytokine receptors, immune recognition receptors, and certain tyrosine kinases. SHP-2 positively regulates signaling events from the activated receptor protein tyrosine kinases, growth factors, hormones, and cytokines and multichain immune recognition receptors. Although recent works suggest that it may have negative (i.e., signal attenuating) in some signaling pathways [Marengòre et al., 1996; Symes et al., 1997].

Previous studies have shown that both, SHP-1 and SHP-2 associate with the members of signal regulation protein family (SIRPs) [Fujioka et al., 1996; Noguchi et al., 1996; Ohnishi et al., 1996; Kharitonenkov et al., 1997; Ochi et al., 1997; Timms et al., 1998; Veillette et al., 1998] and the tyrosine residues in their cytoplasmic domain in the phosphorylated state serve either as binding site for SH2 domains (acting as target) or the dephosphorylation site (acting as the substrate) for the catalytic domain of these phosphatases. SIRPs are members of large inhibitory receptor superfamily constituting a subfamily of trans-

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membrane glycoproteins, which are ubiquitously expressed in a variety of cell types and tissues including heart, brain, liver, placenta, lung, spleen, spinal chord, kidney, etc. [reviewed by Cant and Ullrich, 2001]. They are grouped into two subtypes, SIRPα and SIRPβ, based on differences in their structures. SIRPα subtype has an intracellular domain containing four potential tyrosine phosphorylation sites with the motif pYXXL/V/I in addition to the extracellular domain containing three Ig-like subdomains, while SIRPβ has only an extracellular domain. Currently this family represents more than 15 members including SHPS-1, p84, BIT, MFR, MYD-1, and SIRPα1.

Studies using synthetic phosphotyrosyl peptides derived from SHPS-1 have shown that sites pY^{449} and pY^{473} were the major phosphorylation and binding sites for the SH2 domains of SHP-2, whereas the other two sites, pY^{408} and pY^{432}, were not the phosphorylation and binding sites. However, all the undecapeptides corresponding to four tyrosine phosphorylation sites behaved as the effective in vitro substrate as demonstrated by their dephosphorylation by the catalytic domain of SHP-2 [Takada et al., 1998] In BIT sites, pY^{436}, pY^{460}, and pY^{477} have been shown to the major binding sites for the SHP-2 [Ohnishi et al., 1996]. SIRPα1 (a human homologue of rat SHPS-1 sharing 91% sequence identity) although has been shown to interact with SH2 domains of SHP-2 and SHP-1 [Fujioka et al., 1996; Kharitonenkov et al., 1997; Cant and Ullrich, 2001], yet binding and dephosphorylation sites in this protein are not clear. Apparently all these studies have focused mainly on specificity of SH2 domains of SHP-2 not the SHP-1 in spite of the fact that they are highly homologous in their structures. Further, question arises what about the catalytic PTP domain specificity? There have been some studies using chimeric SHP-1 and SHP-2, which strongly suggest that their catalytic domains too possess specificity in choosing their substrates [Tenev et al., 1997; O'Reilly and Neel, 1998]. Our crystallographic studies with the catalytic domain of SHP-1/SIRPα1 peptide complexes also have indicated that the catalytic domain of SHP-1 specifically recognizes residues at − 4 and further N terminal positions of the phosphotyrosyl peptides [Yang et al., 2000]. To confirm our crystallographic data and address the issue of substrate specificity of the catalytic domain of PTPs, we selected SIRPα1 as the model protein which has been shown to interact with SHP-1 and SHP2 [Fujioka et al., 1996; Kharitonenkov et al., 1997; Cant and Ullrich, 2001]. We carried out kinetic and activation studies with SHP-1 and SHP-2 and their catalytic domain mutants using synthetic phosphotyrosyl peptides derived from SIRPα1.

Based on our results from the kinetic and activation data, we demonstrate that the specificity of the catalytic domain of both of these enzymes is determined by the residues at positions −6 and −7 to the N terminal of the phosphorylated substrate SIRPα1, and further it appears that SIRPα1 may act as both, an upstream activator and substrate for SHP-1, while merely as the downstream substrate of SHP-2 in the signaling events.

MATERIALS AND METHODS

Protein Expression and Purification

The catalytic domains of SHP-1 (245-543), SHP-2 (246-547), the full-length SHP-1 (1-597) and SHP-2 (1-593) were cloned, expressed, and purified as previously described [Liang et al., 1997]. The purified enzymes were concentrated to about 1 mg/ml, aliquoted and stored at 4°C and −20°C for immediate and future usage, respectively.

Peptide Synthesis

Phosphotyrosyl peptides with their amino acid sequences corresponding to the phosphotyrosine sites in the cytoplasmic domain of either SIRPα1 were synthesized and purified to 95% purity by SynPep Corporation (Dublin, California). For the convenience of discussion, the peptides were also named according to the phosphotyrosine sites. Decapeptides pY^{427}, pY^{452}, pY^{469}, and pY^{495} and 13-amino-acid peptide pY^{469+3} were derived from SIRPα1. The sequences of these peptides were pY^{427}, TNDIT-pYADLN; pY^{452}, NNHTEpYASIQ; pY^{469}, EDTLTpYADLD; pY^{495}, PSFSEpYASVQ; and pY^{469+3}, RPNNHTEpYASIQ. The purified peptides were dissolved in 1 ml working buffer (50 mM sodium acetate, pH 5.0, 2 mM EDTA, 2 mM DTT). The final peptide concentrations were determined by amino acid analysis.

Determination of Kinetic Parameters

The phosphatase activity of the catalytic domains of SHP-1 and SHP-2 against the synthetic phosphotyrosyl peptides was determined by the

malachite green method at room temperature [Harder et al., 1994]. The concentration of released phosphate was estimated from the standard curve of OD_{660} vs. $[PO_4^{2-}]$ concentration. The initial velocity of the reaction was calculated as amount of the the released phosphate per minute. The catalytic turn over constant k_{cat} and the Michaelis constant K_m were determined by non-linear least squares fitting of the measured data to the Michaelis-Menton equation, using software, Graphpad prism and Kaleida Graph (3.5).

Activation of SHP-1 and SHP-2

The stimulation of the phosphatase activity of full length SHP-1 and SHP-2 by the SIRPα 1 peptides was measured using pNPP as a substrate as previously described [Pei et al., 1996]. Briefly, enzymes (0.016–0.032 μM), pNPP (10 μM, fixed concentration), and peptides (0.01–350 μM) in a total volume of 50 μl of the buffer, 10 mM HEPES (pH 7.5) containing NaCl (150 mM), DTT (10 mM), EDTA (1 mM), 50% Glycerol (w/v) were allowed to react for 4 min at room temperature. The reaction was quenched by using 0.2 M NaOH (950 μl).The amount of released *p*-nitrophenolate anion was estimated using $\epsilon_{405\ nm}$ 17,800 $M^{-1}cm^{-1}$ to determine the initial velocity. The extent of activation and ED_{50} value (the concentration of the peptide required for half maximal activation) were determined from the plot of relative activity (activity with respect to the basal activity) vs. peptide concentration.

RESULTS

Decapeptides pY^{469} and pY^{495} Derived From SIRPα 1 Are the In Vitro Substrates for the Catalytic Domains of SHP-1 and SHP-2

As discussed earlier, members of the SIRP protein family sharing more than 90% sequence homology in their cytoplasmic domains interact with both SHP-1 and SHP-2 [Ohnishi et al., 1996; Takada et al., 1998; Timms et al., 1998; Veillette et al., 1998; Ohnishi et al., 1999]. The pTyr residues in these molecules either serve as binding site (acting as target) for SH2 domains or dephosphorylation site (acting as a substrate) for the catalytic domain. What is the basis for this different behavior such that the catalytic domains of SHP-1 and SHP-2 specifically choose these protein molecules as their substrate/activator? Moreover in SIRPα1, binding and dephosphorylation sites are not known yet. Therefore, to address these issues, we choose SIRPα1 as a model protein.

We synthesized four phosphotyrosyl decapeptides with sequences flanking four phosphotyrosine sites from its cytoplasmic domain. For the convenience of discussion, the synthetic peptides were named according to the phosphotyrosine sites in SIRPα1, i.e., pY^{427}, pY^{452}, pY^{469}, and pY^{495}. We measured the kinetic parameters for the catalytic domains of SHP-1 and SHP-2 towards these peptides (Table I). The catalytic domain of SHP-1 showed much lower K_m values towards peptides pY^{469} and pY^{495} (80.0 μM and 71.5 μM, respectively) than peptides pY^{452} and pY^{427} (286.0 μM and 312.0 μM, respectively). It also exhibited higher k_{cat}/K_m values towards peptides pY^{469} and pY^{495} (1,772 $s^{-1}mM^{-1}$ and 1,120 $s^{-1}mM^{-1}$, respectively) than peptides pY^{452} and pY^{427} (698 $s^{-1}mM^{-1}$ and 940 s^{-1} mM^{-1}, respectively). These observations indicated that the peptides pY^{469} and pY^{495} are the effective in vitro substrates of the catalytic domain of SHP-1.

Similarly the catalytic domain of SHP-2 also showed lower K_m values (127 and 47.5μM) and higher k_{cat}/K_m values (875 and 822 $s^{-1}mM^{-1}$) towards peptides pY^{469} and pY^{495}, respectively, but to a lower extent than SHP-1. Thus, peptides pY^{469} and pY^{495} also were the effective

TABLE I. Kinetic Parameters for the Catalytic Domains of SHP-1 and SHP-2 Against Phosphotyrosyl Peptides From SIRPα

	SHP-1			SHP-2		
	K_m (μM)	k_{cat} (S^{-1})	k_{cat}/K_m ($S^{-1}mM^{-1}$)	K_m (μM)	k_{cat} (S^{-1})	k_{cat}/K_m ($S^{-1}mM^{-1}$)
EDTLTpYADLD (pY^{469})	80.0	141.7	1,772	127.0	111.1	875
PSFSEp YASVQ (pY^{495})	71.5	80.0	1,119	47.5	39.0	822
TNDITpYADLN (pY^{427})	312.0	217.7	698	160.0	119.2	745
NNHTEpYASIQ (pY^{452})	286.0	268.1	938	304.0	117.2	386
RPEDTLTpYADLDM (pY^{469+3})	327	142.5	436	28.0	40.2	1,436

in vitro substrates for the catalytic domain of SHP-2. In addition, we noticed the K_m and k_{cat}/K_m for the catalytic domain of SHP-2 against peptide pY^{427} were 160.0 μM and 745 $s^{-1}mM^{-1}$, indicating that peptide pY^{427} also behaved as its in vitro substrate. Thus, our kinetic data showed that SIRPα1 decapeptides, pY^{469} and pY^{495} were the effective in vitro substrates for the catalytic domains of both SHP-1 and SHP-2.

Based on amino acid sequence flanking the pY residue in SIRPα1, the four synthetic decapeptides fall into two groups: pY^{427}, pY^{469} and pY^{452}, pY^{495}. The two peptides in each group have very similar sequences at the C-terminal side of residue pTyr. However, only one peptide in each group (pY^{469} and pY^{495}, respectively) is the effective in vitro substrate for SHP-1 and SHP-2. Therefore, substrate specificity of the catalytic domains of SHP-1 and SHP-2 is likely to be mainly determined by the residues N-terminal to pTyr in the peptide substrates.

Catalytic Domains of SHP-1 and SHP-2 Specifically Recognize Residues at the −6 and −7 Positions in Phosphotyrosyl Substrates

Domain swapping studies have shown that the catalytic domains of SHP-1 and SHP-2 possessed different substrate specificity [Tenev et al., 1997; O'Reilly and Neel, 1998]. We did not observe any subtle difference in substrate specificity from our kinetic studies with the catalytic domains of SHP-1 and SHP-2 using synthetic peptides containing residues ranging from −5 to + 4 positions, i.e., pY^{427}, pY^{452}, pY^{469}, and pY^{495}. However we did observe substrate specificity with the peptide containing residues from −7 to + 5 positions, i.e., pY^{469+3} ($^{-7}$RPEDTL-TpYADLDM^{+5}) (Table I).

The activity of catalytic domains of SHP-1 and SHP-2 towards peptide pY^{469+3} was very different from pY^{469}, as shown in the velocity vs. substrate concentration graph (Fig. 1). Compared to decapeptide pY^{469}, the K_m for the catalytic domain of SHP-1 towards the longer trideca-peptide pY^{469+3} increased from 80.0–327.0 μM, and the k_{cat}/K_m decreased from 1,772 $s^{-1}mM^{-1}$ to 436 $s^{-1}mM^{-1}$ (Table I). This suggested that probably peptide pY^{469+3} is not an effective in vitro substrate of the catalytic domain of SHP-1, while the peptide pY^{469} served as its substrate. However, for the catalytic domain of SHP-2, the K_m value for the peptide pY^{469+3} reduced to 28.0 μM from 127.0 μM for peptide pY^{469}, and also the k_{cat}/K_m

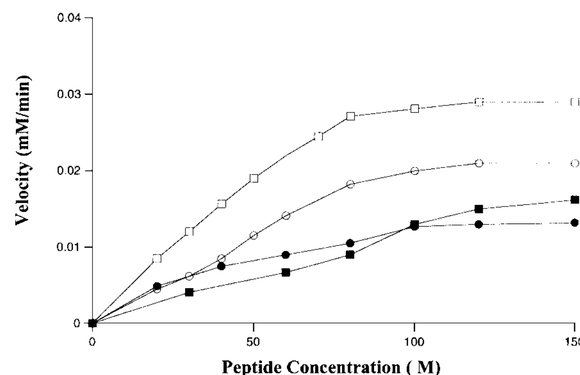

Fig. 1. The graph of initial velocity vs. peptide concentration for the catalytic domain of SHP-1 and SHP-2. The velocity curves for the catalytic domain of SHP-1 towards peptides pY^{469} and pY^{469+3} are shown in open squares (□) and solid squares (■), respectively and for the catalytic domain of SHP-2 by open circles (○) and solid circles (●), respectively.

value substantially increased to 1,436 $s^{-1}mM^{-1}$ from 878 $s^{-1}mM^{-1}$ for pY^{469} (Table I). This suggested that peptide pY^{469+3} is a much better in vitro substrate for the catalytic domain of SHP-2 than pY^{469}. Thus, it becomes clear that by increasing the length of the pY peptide towards its N-terminal side by adding residues at −6 and −7 positions to peptide pY^{469}, the newly generated peptide (pY^{469+3}) behaved as a much better in vitro substrate of the catalytic domain of SHP-2, but no longer remained as a substrate of the catalytic domain of SHP-1. These observations led us to conclude that a remote substrate-recognition site away from the pTyr-binding subpocket is present in the catalytic domains of SHP-1 and SHP-2, which specifically recognized residues at the −6 and −7 positions of phosphotyrosyl peptides.

Activation of Full-Length SHP-1 and SHP-2 by the Phosphotyrosine Decapeptides Derived From SIRPα

As we have seen previously, decapeptides pY^{469}, pY^{495} behaved as better substrates, while pY^{427} and pY^{452} peptides were the poor substrates for the catalytic domains of SHP-1 and SHP-2 (Table I). Increasing the length of the peptide pY^{469} towards N-terminal side clearly made a distinction in behaviors of catalytic domains of SHP-1 and SHP-2. The longer peptide pY^{469+3} acted as a substrate for SHP-2, but no longer remained as a substrate for SHP-1. This suggested to us that peptides, which were the poor substrates for the SHP-1, might be the activator for the SHP-2 and

vice-versa involving the interaction with SH2 domains of SHP-1 and SHP-2. To gain further insight into the interaction of these peptides with full length SHP-1 and SHP-2, we carried out the activation studies in the presence of the peptides and measured the concentration dependent stimulation of the phosphatase activity of the SHP-1 and SHP-2, using pNPP as a substrate (Figs. 2 and 3). SHP-1 was found to be activated by all the peptides, maximally by the pY^{469+3} followed by pY^{469}, pY^{427}, pY^{495}, and pY^{452} and that the concentration required for half maximal activation (ED_{50} values) were 40 μM for pY^{469+3}, 100 μM for pY^{469} and pY^{427}, ≈ 160 μM for pY^{495} and ≈ 300 μM for pY^{452} (Fig. 2). In contrast, SHP-2 was not found to be activated by either of the peptides even at highest concentration of 350 μM (Fig. 3).

DISCUSSION

SIRPs are expressed in varieties of cell types and tissues including heart, brain, liver, placenta, lung, spleen, and spinal chord [reviewed by Cant and Ullrich, 2001]. SHPS-1, the first identified member of the SIRP family, has been shown to be associated with SHP-1 in macrophages [Veillette et al., 1998], but it is unclear whether it is also the physiological substrate of SHP-1. The sites pY^{449} and pY^{473} in SHPS-1 were the major phohorylation and binding site for SHP-2 [Takada et al., 1998]. However, in SIRPα1 (the human homologue of rat SHPS-1 having 91% sequence identity), the binding and dephosphorylation sites are not yet clearly defined. To gain further insight into the understanding of the dephosphorylation and binding sites in SIRPα1 and its specific interaction with SHP-1 and SHP-2, we carried out kinetics and activation studies using phosphotyrosine synthetic peptides containing the amino acid sequences flanking the four tyrosine phosphorylation sites in its cytoplasmic domain.

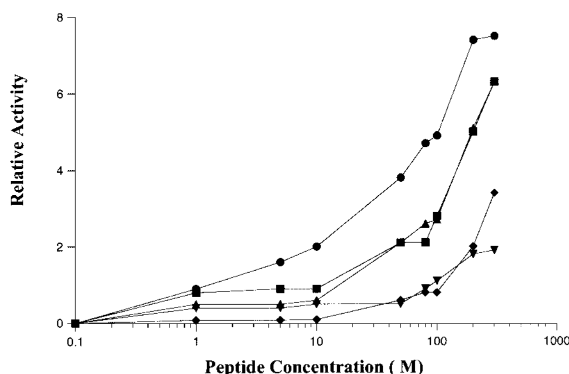

Fig. 2. Activation of full length SHP-1 by various SIRPα1 peptides derived from its cytoplasmic domain. The activation by the peptide pY^{427} is represented by solid square (■), pY^{452} by solid triangle (▲), pY^{469} by inverted solid triangle (▼), pY^{495} by solid diamond (◆), pY^{469+3} by solid circle (●).

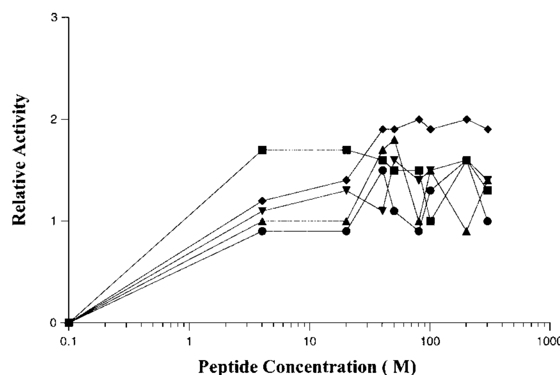

Fig. 3. Activation of full length SHP-2 by various SIRPα1 peptides derived from its cytoplasmic domain. The activation by the peptide, pY^{427} is represented by solid square (■), pY^{452} by solid triangle (▲), pY^{469} by inverted solid triangle (▼), pY^{495} by solid diamond (◆), pY^{469+3} by solid circle (●).

Our results (Table I) showed that peptides, pY^{469} and pY^{495} were the effective substrate for both SHP-1 and SHP-2, while the pY^{452} was a poor substrate for both the phosphatases. Peptide pY^{427} acted as slightly better substrate for SHP-2 compared to SHP-1 as reflected from their K_m values (Table I). These results are supported by the observations made by Takada et al., where they showed that all the four phosphotyrosine-containing peptides from SHPS-1 corresponding to its four phosphorylation sites (pY^{408}, pY^{432}, pY^{449}, and pY^{473}) were the effective in vitro substrates of SHP-2. Furthermore, comparing the amino acid sequences of all the four SIRPα1 peptides, it becomes obvious that they fall into two groups: pY^{427}, pY^{469} and pY^{452}, pY^{495} having very similar sequences on C-terminal side (+ side) to pY. Thus, now it appears that the substrate specificity of the catalytic domain of both of these phosphatases is primarily determined by the amino acid residues residing on N-terminal side (−side) to pY. This is in consistent with our previously published results of the crystal structures of peptides, pY^{469} and pY^{495} complex with SHP-1, where it has been proposed that the substrate specificity of the catalytic domain of SHP-1 is determined by the residues at −4 and further N-terminal positions in the peptide/

protein substrates [Yang et al., 2000]. To further address this question, we examined a longer tridecapeptide (pY^{469+3}) which contained residues up to -7 positions from the pY. We observed that this longer peptide behaved remarkably different with SHP-1 and SHP-2 (Fig. 1). It acted as the substrate for SHP-2 only (K_m 28 μM) (Table I).With SHP-1, there was a 12-fold increase in the K_m value, whereas k_{cat}/K_m value was decreased by ~3.5-folds (Table I) indicating that perhaps pY^{469+3} is not the substrate for SHP-1, and the substrate binding pocket lies remotely from the p-Tyr binding site in the catalytic domains, which specifically recognizes residues at the -6 and -7 positions of the peptide substrate. This holds true for another known substrate of SHP-1, CD22 [Blasioli et al., 1999], which is a B cell specific transmembrane lectin with 6 phosphorylation sites and has previously been shown to be involved in the B cell signaling pathways. We obtained similar results with CD22 peptides (data not shown), where it was also found that longer peptide pY^{837+2} having amino acid residues from the N-terminal side was a much better substrate for SHP-2 than SHP-1 compared to the shorter decapeptide (pY^{837}). Thus, based on the kinetic data, we show that catalytic domains of both SHP-1 and SHP-2 have distinct substrate recognition and binding sites, and the substrate specificity is mainly determined by the residues present at -6 and -7 positions of peptide/protein substrates. In the activation studies, we have shown that while SHP-1 is more or less is activated by all the peptides, the SHP-2 could not be activated by any of the peptides (Figs. 2 and 3). The maximum activation was by pY^{469+3} ($ED_{50} \approx 42$ μM) followed by pY^{469} and pY^{427} ($ED_{50} \approx 127$ μM), pY^{495} ($ED_{50} \approx 160$ μM) and pY^{452} ($ED_{50} > 350$ μM). From these data, it becomes apparently clear that the site pY^{469} of the SIRPα1 is both, binding and the dephosphorylation site for SHP-1, while for SHP-2 it is mainly the dephosphorylation site. In other words, SIRPα1 may act as both a substrate and an activator for SHP-1, while remaining as the downstream substrate for SHP-2 in signal transduction pathways. Studies using phosphotyrosine containing peptides with other highly homologous members of SIRP family, SHPS-1 and BIT [Ohnishi et al., 1996; Takada et al., 1998; Ohnishi et al., 1999] have shown that SHPS-1 pY^{449}, pY^{477} (corresponding site in SIRPα1 are pY^{469} and pY^{495}) and BIT pY^{436}, pY^{460}, and pY^{427} (corresponding sites in SIRPα1, pY^{427}, pY^{452}, and pY^{469}) were the major SHP-2 SH2 binding sites, and the peptides containing these sites were also shown to be able to activate SHP-2 in vitro. In contrast to these results, we did not observe any activation of SHP-2 by the SIRPα1 phosphotyrosine containing peptides, instead SHP-1 was found to be activated by all these peptides. This activation of SHP-1 by the peptides is supported by the previous studies, where it has been shown that SH2 domains of SHP-1 bind the consensus sequence with the motif I/VXpYXXL [Sugimoto et al., 1993; Burshtyn et al., 1997; O'Reilly and Neel, 1998] and three of the most potent SIRPα1 peptides, pY^{469+3} ($ED_{50} \approx 42$ μM), pY^{469} and pY^{427} ($ED_{50} \approx 127$ μM) had L at position $+3$ from the pY. The other two peptides (pY^{452} and pY^{495}) which had either I/V at this position were less potent in activation as evident from their ED_{50} values (≈ 160 μM for pY^{452} and > 350 μM for pY^{495}). The consensus sequence for the SHP-2 SH2 binding site has been partly defined to be XXpYI/V-nonbasic-V/I/L/P-nonbasic-hydrophobic [Sugimoto et al., 1993; Burshtyn et al., 1997; Kuriyan and Cowburn, 1997; O'Reilly and Neel, 1998]. None of the SIRPα1 peptides studied here had the I/V at position $+1$. Perhaps because this SHP-2 was not activated by any of the peptides.

In summary, our results demonstrated that substrate binding pocket in the protein or peptide substrates is remotely present from the pTyr binding site in the catalytic domains of both SHP-1 and SHP-2. This substrate binding pocket specifically recognizes residues at the -6 and -7 positions relative to pTyr. SIRPα1 seems to be acting both an upstream activator and substrate for SHP-1, while merely as the downstream substrate for SHP-2. The results from our work may be extended to find out specificities of other phosphatases using similar experiments. Furthermore, as both SHP-1 and SHP-2 specifically recognizes residues at the -6 and -7 positions form the phosphorylated tyrosine, this knowledge could be utilized in designing the specific inhibitors of these phosphatases.

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