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Kinetic Comparison of the Catalytic Domains of SHP-1 and SHP-2

Tianqi Niu,[1] **Xiaoshan Liang,**[1] **Jian Yang,**[1] **Zhizhuang Zhao,**[2] **and G. Wayne Zhou**[1]*

[1]Program in Molecular Medicine, UMASS Medical Center, Worcester, Massachusetts 01605
[2]Department of Medicine, Vanderbilt University, Nashville, Tennessee 37232

Abstract The phosphatase activity of SH2-containing protein tyrosine phosphatase (SHP) is inhibited by its SH2 domains and C-terminal tail. In order to determine the inhibitory effects of the SH2 domains and C-terminal tail, we have expressed and purified the catalytic domains of SHP-1 and SHP-2, and the SH2 domain truncated SHP-1 and SHP-2. We have then measured their kinetic parameters using p-nitrophenyl phosphate (p-NPP) and phosphotyrosine (pY) as substrates under the same experimental conditions. The results indicate that the pH-dependent profiles of SHP-1 and SHP-2 are mainly determined by their catalytic domains. Both enzymes have maximum activity at pH 5.0. In addition, the phosphatase activity of different forms of SHP-1 and SHP-2 decreases as the salt concentration increases. Without SH2 domains, both SHP-1 and SHP-2 are no longer inhibited by their C-terminal tails. However, the C-terminal tail of SHP-1 can further prevent the salt inhibition of the phosphatase activity. Under the same experimental conditions, the catalytic domain of SHP-1 is two times more active than the catalytic domain of SHP-2. J. Cell. Biochem. 72:145–150, 1999.

Key words: SHP; catalytic domain; PTPases; PTKases

Protein tyrosine phosphatases (PTPases), together with the protein tyrosine kinases (PTKases), regulate the critical phosphorylation levels of signal transduction pathways and control cell growth, proliferation, and differentiation [Walton and Dixon, 1993; Hunter, 1995; Neel and Tonks, 1997]. SHP-1 and SHP-2 belong to the SH2-containing protein tyrosine phosphatase family (SHPs) which contains two tandem SH2 domains at the N-terminus followed by a single catalytic domain and a C-terminal tail. SHP-1 and SHP-2 are two of the most extensively studied PTPases [D'Ambrosio et al., 1995; Klingmüller et al., 1995; Yamauchi et al., 1995; Zhao et al., 1995a; Plas et al., 1996; Eck et al., 1996; Ono et al., 1997; Hof et al., 1998]. SHPs are cytosolic enzymes and are essentially inactive within the resting cells. Upon cell stimulation, they can translocate from the cytosol to the plasma membrane by binding to tyrosine-phosphorylated receptors through their SH2 domains and become activated in the process. SHP-1 is expressed predominantly in hematopoietic cells, whereas, SHP-2 is broadly expressed within cells. The direct down stream substrates for SHP-2 were identified as transmembrane glycoproteins SHPS-1 and SIRPs [Fujioka et al., 1996; Kharitonenkov et al., 1997]. Although the catalytic activities and the modes of regulation of different SHPs appear similar, their functions are opposite within the cells. SHP-1 usually serves as a negative regulator in the proliferation of hematopoietic cells, whereas SHP-2 is positively involved in the growth factor mediated signal transduction and promote cell proliferation [Streuli, 1996].

Previous kinetic studies have shown that the phosphatase activity of SHPs is inhibited by their own SH2 domains and their C-terminal tails [Sugimoto et al., 1993; Townley et al., 1993; Zhao et al., 1993, 1994]. Occupancy of the SH2 domains by phosphotyrosine peptides [Lechleider et al., 1993; Pluskey et al., 1995], removal of the SH2 domains [Sugimoto et al., 1993; Townley et al., 1993], and removal of the C-terminal [Zhao et al., 1993, 1994] would significantly improve the phosphatase activity of SHPs. In order to determine the inhibitory effect of the SH2 domains and, especially, the

*Correspondence to: G. Wayne Zhou, Program in Molecular Medicine, UMASS Medical Center, 373 Plantation Street, Worcester, MA 01605.

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inhibitory effect of C-terminal tail, we have carried out the kinetic studies of the catalytic domains of SHP-1 and SHP-2. For comparison, we also remeasured the kinetic parameters of full length SHP-1, SHP-2, and their corresponding SH2 domain truncated forms under the same experimental conditions.

MATERIALS AND METHODS

Expression of Proteins

The SH2 domain truncated SHP-1 (Δ1C, amino acids 210–597), the SH2 domain truncated SHP-2 (Δ2C, amino acids 192–593), the catalytic domain of SHP-1 (D1C, amino acids 245–543), and the catalytic domain of SHP-2 (D2C, amino acids 246–547) were cloned and expressed in *E. coli* BL21 (DE3) cell line. Those different recombinant forms of SHP-1 and SHP-2 are summarized in Table 1. The catalytic domains in SHP-1 and SHP-2 correspond to the catalytic domain in the crystal structure of PTP1B [Barford et al., 1994]. The detail PCR cloning procedure for the catalytic domain of SHP-1 has been reported [Liang et al., 1997]. Same procedure was used to clone and express the catalytic domain of SHP-2. The DNA fragment encoding 302 amino acids from Gly 246 to Tyr 547 of SHP-2 was PCR synthesized with *pfu* polymerase (Strategene, La Jolla, CA) using plasmid pT7-PTP2C that contains the full length SHP-2 as the template [Zhao et al., 1994]. The N-terminal primer (5′-GGAATTC-CATATGGGCTTTTGGGAAGAATTTGAG-3′) contains an Nde I restriction enzyme site followed by 21 nucleotides from Gly 246. The C-terminal primer (3′-TTTCCCGTGCTTATAT-GTTTATAATTCATAATTCGAAGGG-5′) contains a Hind III site, a stop codon followed by 21 nucleotides from the anti-strand of Tyr 547. Other cloning and expression procedures were the same as those for D1C expression.

Protein Purification

All phosphatases were purified using the modified purification protocol [Liang et al., 1997]. In summary, the cell lysate was first purified by running through an FFQ column (Q Sepharose fast flow column from Pharmacia, Gaithersburg, MD). The flow-through was then directly loaded onto the HDBP (L-Histidyldiazobenzylphosphonic acid) column. The HDBP column was then washed with buffer QA (25 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, 1 mM EDTA) to get rid of other proteins. The phosphatases were eluted from the HDBP-column using a 0 to 1 M NaCl gradient in buffer QA. The purity of the fractions were examined with 12% SDS-PAGE gel. The pure fractions were then combined, concentrated, and stored at -20°C, in the presence of 10% glycerol.

Determination of Kinetic Parameters

The activity assay was performed at room temperature (23 ± 0.5°C) under different pH, using the published protocol [Zhao et al. 1993]. The total volume for the dephosphorylation reaction was 100 µl, containing both enzyme and substrate in the working buffer (50 mM NaOAc-HOAc, pH 5.0, 2 mM EDTA, 2 mM DTT, 40% glycerol). For substrate p-nitrophenyl phosphate (p-NPP), the reaction was quenched with the addition of 900 µl of 0.2 M NaOH. The concentration of the released product, p-nitrophenol, was measured by OD_{410} with the extinction coefficient of 17.8 per mM. For each protein, the progress curve (absorbance versus time) was measured to determine the experimental conditions so that the progress curve

TABLE I. Summary of Different Recombinant Forms of SHP-1 and SHP-2

Constructs	Source	Sequence range	Diagram representation
D1C	SHP-1	245–543	PTP domain
D2C	SHP-2	246–547	PTP domain
Δ1C	SHP-1	210–597	PTP domain
Δ2C	SHP-2	192–593	PTP domain
F1C	SHP-1	1–597	SH2 SH2 PTP domain
F2C	SHP-2	1–593	SH2 SH2 PTP domain

has linear range within the initial 5 min. After determining the appropriate protein concentration, the dephosphorylation reaction were setup under different substrate concentrations, ranging from 0.1 to 5 K_m. The reactions were then quenched by NaOH at 5 min. The concentration of the released product was determined by absorbance at 410 nm and the initial reaction rate for different substrate concentration was calculated. From the double reciprocal plot, the kinetic parameters for different proteins were determined. The kinetic parameters were average of 20 experiments.

For the activity assay against substrate phosphotyrosine (pY), the malachite green method was used to measure the releases of inorganic phosphate [Harder et al., 1994]. At first, the standard OD_{660} versus $[PO_4^{2-}]$ curve was measured using malachite green reagent as the standard for the determination of the released phosphate concentration. The reaction procedure was the same as above except that malachite green mix (three volumes of 0.045% malachite green hydrochloride in water and 1 volume of 4.2% ammonium molybdate in 4 M HCl) was used to quench the reaction. The absorbance of the reaction mixture was measured at 660 nm. The released phosphate concentration was determined from the standard OD_{660} vs. $[PO_4^{2-}]$ curve.

RESULTS AND DISCUSSION

Purification of Proteins

Δ1C, Δ2C, D1C, and D2C were expressed in *E. coli* and purified by anion exchange chromatography and affinity chromatography. The expression and purification protocol was published by Liang et al. [1997]. The purified proteins have more than 90% purity as shown in Figure 1. D1C and D2C are shown in lanes 1 and 2. Δ1C and Δ2C are shown in lanes 3 and 4. Apparently, SHP-2 has a higher molecular weight than SHP-1.

pH Dependence of the Phosphatase Activity of D1C and D2C

The pH dependence of the phosphatase activities of the catalytic domains D1C and D2C was examined using p-NPP as the substrate. The pH rate profiles of both D1C and D2C are bell shaped, with the maximal activity at pH 5.0 (Fig. 2). The pH profiles are very similar to those of SHP-1 [Zhao et al., 1993] and SHP-2

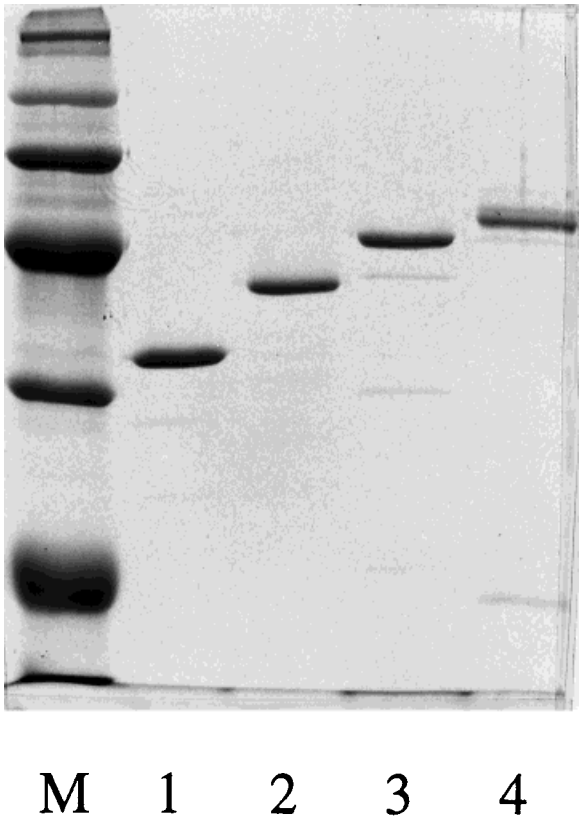

Fig. 1. Characterization of D1C, D2C, Δ1C, and Δ2C by 12% Tris-glycine-SDS polyacrymide gel electrophoresis. M is protein marker with the molecular weights 200 kd, 97.4 kd, 68 kd, 43 kd, 29 kd, and 18.4kd ranging from the top to the bottom. From **lane 1** to **lane 4** are D1C, D2C, Δ1C, and Δ2C, respectively.

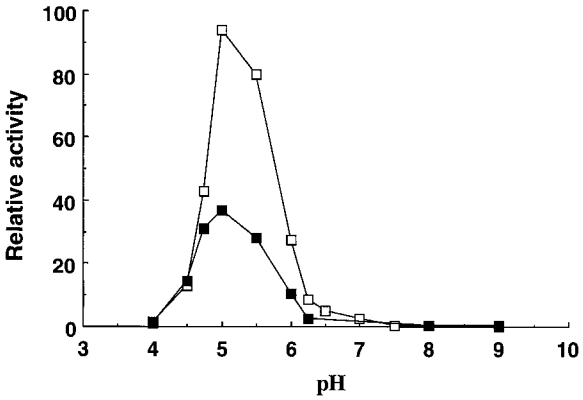

Fig. 2. The pH-rate profiles of the D1C and D2C. D1C and D2C are represented by □ and ■ respectively.

[Sugimoto et al., 1993], which are also bell-shaped and have the optimal pHs for activity at pH 4.6 and 5.6 for SHP-1 and SHP-2, respectively. Studies by Pei et al. [1993] indicate that the optimal pH for SHP-1 is at 5.5–5.7. It is probably due to the different experimental con-

ditions that the optimal pH for SHP-1 is measured slightly different in different laboratories. The optimal pHs for D1C and D2C are not very far from those of SHP-1 and SHP-2, respectively. Therefore, the pH-rate profiles of SHP-1 and SHP-2 are probably mainly determined by their catalytic domains. The SH2 domains and the C-terminal tail do not change the pH-rate profiles of SHP-1 and SHP-2. The phosphatase activity of either SHP-1 or SHP-2 drops dramatically on both sides of pH 5.0. For both SHP-1 and SHP-2, very little activity is left if the pH is lower than 4.3 or higher than 6.3. In addition, D1C is more active than D2C in the pH range of 4.3 to 6.3. At the optimal pH, the phosphatase activity of D2C is only about 40% as that of D1C.

Salt Dependence of Phosphatase Activity of D1C and D2C

Salt concentration in the reaction system significantly affects the phosphatase activity of PTPases [Pei et al., 1994; Zhang, 1995]. Usually, the phosphatase activity is decreased at high salt concentration. To examine the effects of salt to the phosphatase activities of D1C and D2C, we have determined their kinetic parameters for the dephosphorylation reaction under different salt concentration. As a comparison, the kinetic parameters for Δ1C and Δ2C were also measured under the same salt concentrations. All kinetic experiments were performed against p-NPP at pH 5.0, 23°C ± 0.5°C. The salt dependence of their kinetic parameters are plotted in Fig. 3. Respectively, the k_{cat} decreases and the K_m increases for all the four proteins as the salt concentration increases. As the NaCl concentration is increased from 0 to 0.5 M, the K_ms for D2C and Δ2C are increased by approximately three-fold, respectively, whereas for D1C and Δ1C, the K_ms are increased by about 11-fold and 13-fold, respectively. The k_{cat}s for D2C and Δ2C are almost the same at the same salt concentration. The k_{cat} for D1C is higher than the k_{cat} of Δ1C when the salt concentration is less than 0.1 M; and is lower than the k_{cat} of Δ1C when the salt concentration is more than 0.1 M. As the salt concentration is increased from 0 to 0.15 M, the k_{cat} decreases by about four-fold for D1C, whereas only two-fold for Δ1C. This suggests that C-terminal tail could prevent the salt induced inhibition in D1C, but not D2C. At each salt concentration, the k_{cat} of

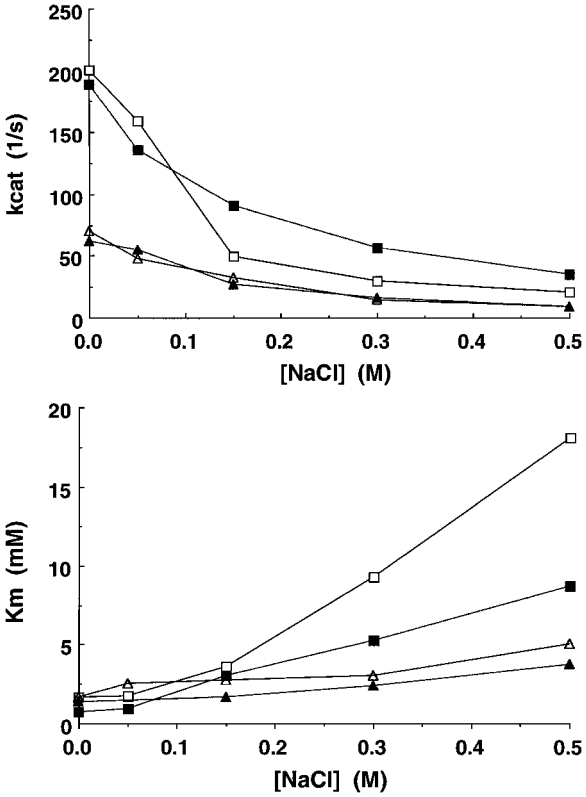

Fig. 3. The salt effects on k_{cat} and K_m for D1C, D2C, Δ1C, and Δ2C. D1C, D2C, Δ1C, and Δ2C are represented by □, ■, △, and ♦, respectively.

D1C is higher than the k_{cat} of D2C. This also indicates that D1C is more active than D2C.

D1C Has Higher Phosphatase Activity Than D2C

As illustrated above, the phosphatase activity of D1C is higher than that of D2C at the same pH or salt concentration. Zhao et al. [1995b] and Sugimoto et al. [1993] reported that SHP-1 has higher activity than SHP-2. Dechert et al. [1995] observed only modest differences between SHP-1 and SHP-2 and between Δ1C and Δ2C towards a group of synthetic phosphotyrosyl peptides. Since those experimental conditions are different from ours, it is not easy to compare the relative activities of different forms of SHP-1 and SHP-2. In order to put the kinetic studies on the same scale, we re-measured the kinetic parameters of full length SHP-1 and SHP-2, Δ1C and Δ2C, and D1C and D2C at pH 5.0, using p-NPP as the substrate. The results are summarized in Table 2. The specific activities of D1C and Δ1C are about three times higher than those of D2C and Δ2C, whereas the K_ms are almost similar for

TABLE II. Kinetic Constant Comparison for the Hydrolysis of p-NPP and pY by PTPase at pH 5 and 23 ± 0.5°C

Substrate	Enzyme	k_{cat} [S^{-1}]	K_m [mM]	k_{cat}/K_m [$mM^{-1}\ S^{-1}$]
p-NPP	D1C	200.9	1.66	121.0
	Δ1C	188.6	0.69	273.3
	F1C	5.8	0.70	8.3
	D2C	70.4	1.63	43.2
	Δ2C	61.1	1.40	43.6
	F2C	3.0	1.05	2.9
PY	D1C	63.8	0.66	96.7
	D2C	15.9	2.66	6.0

different forms of SHP-1 and SHP-2. These suggest that the active site rearrangement in SHP-1 is more efficient than that in SHP-2. The higher phosphatase activity of SHP-1 than SHP-2 have also been observed when phosphotyrosine was used as the substrate (Table 2).

Function of the C-terminal Tail of SHP-1 and SHP-2

The full length SHP-1 and SHP-2 were regulated by both the SH2 domains at the N-terminal and the C-terminal tail after the catalytic domain. Removal of the SH2 domains at the N-terminal or the C-terminal tail can increase the phosphatase activity of both SHP-1 and SHP-2 [Sugimoto et al., 1993; Townley et al., 1993; Zhao et al., 1993, 1994]. However, we still do not know where the anchor site for the C-terminal tail is. Does the inhibitory function of the C-terminal tail need the cooperation of the N-terminal SH2 domains? In other words, after removing the N-terminal SH2 domains, will the C-terminal tail still inhibit the phosphatase activity? Our experiment results (Table 2) show the k_{cat}s are the same for D1C and Δ1C pair at low salt concentration, and so are k_{cat}s for D2C and Δ2C pair. These indicate that D1C and Δ1C have the same phosphatase activities, and D2C and Δ2C also have same phosphatase activity. Therefore, the C-terminal tail has no further inhibiting effects on the kinetic activity of the SHPs after removing the SH2 domains. However, it is still not clear how the C-terminal tail cooperates with the SH2 domains to regulate the activities of SHPs. A crystal structure of the full length SHP-1 or SHP-2 might provide us an insight of the cooperated regulation of SHPs.

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