Inhibition of the Activity of Protein Tyrosine Phosphatase 1C by Its SH2 Domains

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ABSTRACT: Full-length protein tyrosine phosphatase 1C (PTP1C), the catalytic domain of PTP1C (ΔPTP1C), and the N-terminal SH2 domain truncated PTP1C (ΔNPTP1C) were overexpressed in *Escherichia coli* and purified to near homogeneity. Various phosphorylated states of the synthetic phosphotyrosyl peptide TRDIYETDYYRK (IRP), corresponding to the major insulin receptor autophosphorylation sites, were used as substrates for the PTPs. There was no indication for selective dephosphorylation of any of the three phosphotyrosyl residues from the triphosphotyrosyl IRP. Kinetic studies were carried out using all seven different phosphotyrosyl IRPs. Saturation kinetics were observed for PTP1C using the triphosphotryosyl IRP only. In contrast, for ΔPTP1C, saturation was achieved for all seven phosphotyrosyl IRPs. The best substrate for ΔPTP1C was the triphosphotyrosyl IRP possessing a K_m of approximately 1.6 μM, about 3–4-fold lower than either the mono- or diphosphotyrosyl IRPs. However, in contrast to ΔPTP1C, PTP1C had a 22-fold lower affinity for triphosphotyrosyl IRP. Furthermore, deletion of a single N-terminal SH2 domain increased the affinity of the enzyme for the triphosphotyrosyl IRP to a value similar to that obtained with ΔPTP1C. The pH optima for all three enzyme constructs were very similar and could not account for the observed change in substrate affinity between the three enzymes. These results suggest that the SH2 domain of PTP1C exerts an inhibitory effect on its PTP activity.

The phosphorylation of proteins on tyrosyl residues plays a central role in the regulation of a variety of cellular processes (Hunter, 1986, 1989; Ulrich & Schlessinger, 1990; Glenney, 1992). The phosphorylation status of a protein in the cell reflects a balance between two competing processes, namely, phosphorylation catalyzed by protein tyrosine kinases (PTKs)[1] and dephosphorylation mediated by protein tyrosine phosphatases (PTPs). It is currently known that the PTPs are a large family of enzymes specific for the dephosphorylation of phosphotyrosyl residues (Fischer *et al.*, 1991; Saito & Streuli, 1991; Brautigan, 1992; Pot & Dixon, 1992). PTPs are divided into two groups. The first group of PTPs are transmembrane molecules having a variable extracellular domain (in some cases they may possess the hallmarks of a ligand binding domain), as well as one or two intracellular catalytic domains. The members of this group include CD45, LAR, and PTP α, β, γ, δ, ε, and ζ. The second group consists of cytoplasmic enzymes having a single catalytic domain and a variable amino- or carboxyl-terminal regulatory domain. Members of this group of PTPs are typified by PTP1B and include TCPTP, PTPH1, PTP1C, PTP MEG, PTP2C, PTP-PEP, and PTP-PEST. Among these cytoplasmic enzymes, both PTP1C and PTP2C are unique in having two amino-terminal SH2 domains (Shen *et al.*, 1991; Adachi *et al.*, 1992; Freeman *et al.*, 1992; Hiraga *et al.*, 1992; Matthews *et al.*, 1992; Plutzky *et al.*, 1992; Yi *et al.*, 1992; Ahmad *et al.*, 1993; Feng *et al.*, 1993; Vogel *et al.*, 1993).

SH2 domains were originally identified in the *src* family of PTKs; they are noncatalytic and consist of about 100 amino acids conserved among a variety of signal-transducing proteins (Cantley *et al.*, 1991; Koch *et al.*, 1991; Pawson & Gish, 1992; Walksmann *et al.*, 1993). SH2 domains have been found in a diverse array of proteins, some having catalytic activities like PLC-γ, GAP, pp60$^{c\text{-}src}$, PTP1C, and PTP2C, while others such as GRB2 and p85 of PI3 kinase have no apparent intrinsic enzymatic activity. It has been shown that SH2 domains bind to unique phosphotyrosine-containing regions of various growth factor receptors and other signaling molecules (Carpenter *et al.*, 1993; Skolnik *et al.*, 1993). This binding is thought to bring about key protein–protein interactions that are necessary for transducing the signals that were generated at the cell surface in response to ligands. Although this particular function of the SH2 domain has been studied in detail, it is not known what other functions, if any, the SH2 domains play a role in.

In this paper, we describe the overexpression and purification of full-length, catalytic domain, and N-terminal SH2 domain truncated PTP1Cs. We show, using phosphotyrosyl peptides corresponding to the major site of insulin receptor autophosphorylation, that the SH2 domain exerts an inhibitory effect on the activity of PTP1C.

EXPERIMENTAL PROCEDURES

Materials and Methods. The seven phosphotyrosyl peptides of the sequence TRDIYETDYYRK (IRP) corresponding to residues 1142–1153 of the human insulin receptor were chemically synthesized and purified by Peninsula Laboratories, Inc. (Belmont, CA). Amino acid composition analysis, mass spectrometry, and solid-phase sequencing confirmed the

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[1] Abbreviations: IRP, insulin receptor peptide (TRDIYETDYYRK); PTP, protein tyrosine phosphatase; PTK, protein tyrosine kinase; LAR, leukocyte antigen-related; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; BAEE, N^{α}-benzoyl-L-arginine ethyl ester; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCPTP, T-cell PTP; PLC-γ, phospholipase C-γ; GAP, GTPase activating protein; PI3 kinase, phosphoinositide 3-kinase; IRS-1, insulin receptor substrate 1; GRB2, growth factor receptor binding protein 2.

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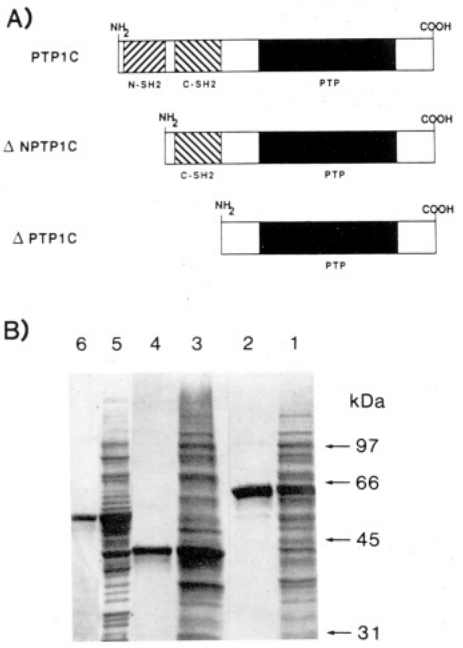

FIGURE 1: (A) Schematic representation of PTP1C, ΔPTP1C, and ΔNPTP1C constructs. (B) SDS–PAGE of purified PTP1C, ΔPTP1C, ΔNPTP1C, and the soluble extract. An aliquot of the crude soluble extracts and the purified enzymes for PTP1C (lanes 1 and 2), ΔPTP1C (lanes 3 and 4), and ΔNPTP1C (lanes 5 and 6) were subjected to SDS–10% PAGE and stained with Coomassie blue. The molecular masses of the standard proteins are indicated in kilodaltons.

sequences of the peptides (Ramachandran *et al.*, 1992). L-Histidyldiazobenzylphosphonic acid agarose was obtained from Sigma.

PTP1C Constructs. PTP1C (amino acids 1–597), ΔNPTP1C (amino acids 108–597), and ΔPTP1C (amino acids 209–597) of the clone PTP1C2 (Shen *et al.*, 1991) were cloned in-frame at the *Bam*HI site of the pET-3C expression vector (Studier *et al.*, 1990). This resulted in 17, 9, and 9 additional amino acids at the N-terminus of PTP1C, ΔNPTP1C, and ΔPTP1C respectively. These constructs were transformed into *Escherichia coli* BL21 (DE3), which carries the T7 polymerase gene controlled by the *lacUV5* promoter. For a schematic representation of the three constructs see Figure 1A.

Purification of PTP1C. E. coli culture was grown in 200 mL of 2× YT medium containing ampicillin (100 μg/mL) at 37 °C to an A_{600} of 0.6–0.8. The temeprature of the culture was lowered to 26 °C and induced with 25 μM isopropyl β-D-thiogalactoside for 16 h. The cells were harvested by centrifugation, resuspended in 20 mM Hepes (pH 7.4) containing 1 mM EDTA, 5 mM DTT, 1 mM PMSF, 1 mM BAEE, and 10 μg/mL each leupeptin, aprotinin, and 100 μg/mL lysozyme. The suspension was incubated on ice for 15 min and sonicated twice for 15 s with 1-min intervals. The lysed suspension was centrifuged at 14500*g* for 15 min. The supernatant was applied to a Mono Q column equilibrated with 20 mM imidazole (pH 7.2), 0.1 mM EDTA, and 0.2% 2-mercaptoethanol (buffer A). The enzyme was eluted using a NaCl gradient. The peak activity fractions were pooled and diluted 4-fold with buffer A and applied to a column of L-histidyldiazobenzylphosphonic acid agarose and eluted with a NaCl gradient. Column fractions were assayed using *p*-nitrophenyl phosphate (Pot *et al.*, 1991).

PTP Assays. For the kinetic studies PTP1C, ΔPTP1C, and ΔNPTP1C assays were carried out at 25 °C in a buffer containing 25 mM imidazole, pH 7.0, 45 mM 2-mercaptoethanol, 20 μg/mL BSA, and varying concentrations of phosphotyrosyl IRPs. Reactions were initiated upon enzyme addition and terminated with ice-cold trifluoroacetic acid to a final concentration of 0.2% (v/v). The final solution was filtered through 0.22-μm Millex-GV4 filters, with substrates and products separated by HPLC as previously described (Ramachandran *et al.*, 1992). All experiments were carried out at least twice and the assays were carried out in duplicate. The kinetic constants presented are the average data as indicated in the legends.

RESULTS

Overexpression and Purification of PTP1C. The full-length PTP1C was initially expressed as a maltose-binding fusion protein and purified using a maltose agarose column. Although substantial purification was achieved, the fusion protein had very low activity. Therefore, PTP1C, ΔPTP1C, and ΔNPTP1C (Figure 1A) were expressed using the T7 promoter. After induction with IPTG, a major protein of the expected molecular weight was obtained and the protein was purified to near homogeneity from the soluble lysate using two-step column chromatography (Table I). SDS–PAGE analysis of the purified proteins indicated a single band of the correct molecular weight for all three constructs (Figure 1B).

Specificity of PTP1C for Triphosphotyrosyl IRP. We determined whether or not PTP1C possessed any site specificity for triphosphotyrosyl IRP dephosphorylation and what role, if any, the SH2 domains played in altering this specificity. On the basis of the total phosphotyrosine content for each of the three positions of the diphosphotyrosyl IRP product, no preference could be ascribed to either PTP1C or ΔPTP1C. Each of the three positions contained similar ratios of phosphorylated to nonphosphorylated tyrosine residues (Figure 2), suggesting that the SH2 domains are not involved in regulating dephosphorylation site specificity. This is in contrast to both CD45 and LAR, which exhibited preferences for dephosphorylating tyrosine at position 5, and PTP1B, which preferred positions 9 and 10 of the triphosphotyrosyl IRP (Ramachandran *et al.*, 1992).

Kinetic Analysis of PTP1C and ΔPTP1C Using IRPs. The kinetics of PTP1C and ΔPTP1C using phosphotyrosyl IRPs are shown in Figure 3. The rate of dephosphorylation for 10-monophosphotyrosyl IRP is representative of all three monophosphorylated IRPs, as is the 5,9-diphosphotyrosyl IRP for all the diphosphorylated substrates. Interestingly, the substrate versus velocity curves for PTP1C never approached saturation when mono- and diphosphotyrosyl IRPs were used as substrates (Figure 3A). Furthermore, the rate of dephosphorylation for these substrates was still linear even when substrate concentrations were above 800 μM. However, using triphosphotyrosyl IRP, the rate of dephosphorylation was linear at low substrate concentration; maximal velocity was achieved at approximately 80 μM (Figure 3A). The substrate versus velocity curves for ΔPTP1C approached saturation around 20 μM for both the mono- and diphosphotyrosyl IRPs and 4 μM for the triphosphotyrosyl IRP (Figure 3B). By use of double-reciprocal plots, the kinetic constants (K_m, V_{max}, and k_{cat}/K_m) of ΔPTP1C were calculated for all seven phosphotyrosyl IRPs (Table II). The K_m for ΔPTP1C using the triphosphotyrosyl IRP was approximately 3–4-fold lower and the V_{max} about 1.5–4-fold higher than the other phosphotyrosyl IRPs. This resulted in a 7–10-fold higher catalytic efficiency ratio (k_{cat}/K_m) for the triphosphotyrosyl IRP. All of the kinetic constants calculated for the mono- and diphosphotyrosyl IRPs were comparable (Table II). The K_m for PTP1C is approx-

Table I: Purification Procedure of PTP1C, ΔPTP1C, and ΔNPTP1C[a]

	PTP1C		ΔPTP1C		ΔNPTP1C	
enzyme pool	total protein (mg)	specific activity (μmol min^{-1} mg^{-1})	total protein (mg)	specific activity (μmol min^{-1} mg^{-1})	total protein (mg)	specific activity (μmol min^{-1} mg^{-1})
extract	72.5	0.403	62.6	1.24	97	0.431
Mono Q pool	9.6	3.78	6.58	9.37	6.67	5.54
L-histidyl pool	1.68	6.18	1.41	24.8	1.16	17.85

[a] PTP1C, ΔPTP1C, and ΔNPTP1C were purified 15-, 20-, and 40-fold, respectively, using Mono Q and L-histidyldiazobenzylphosphonic acid agarose columns as described in the text. The data are representative of a typical purification procedure.

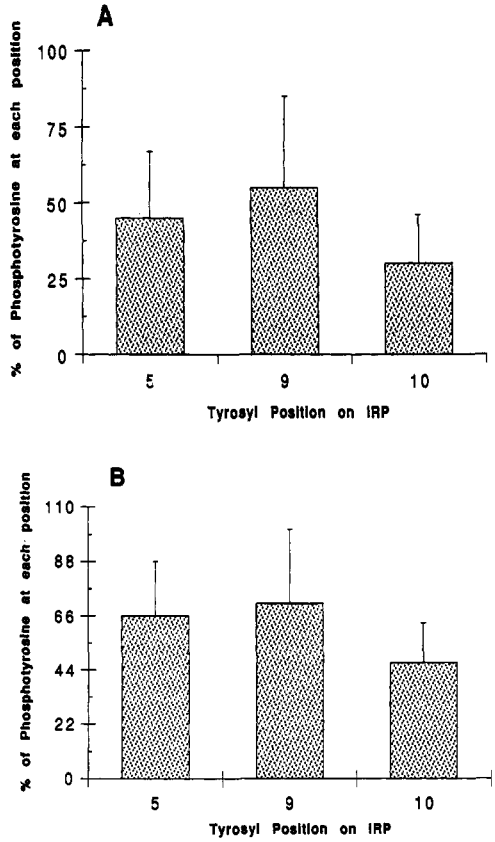

FIGURE 2: Phosphotyrosine content of the diphosphotyrosyl IRP product. Triphoshpotyrosyl IRP (22.7 μM) was incubated with 1.8 μg/mL PTP1C (panel A) or 0.85 μg/mL ΔPTP1C (panel B) in 25 mM imidazole (pH 7.2), 1 mM EDTA, and 45 mM 2-mercaptoethanol for 10 and 1 min, respectively. Under these conditions, less than 5% of the triphosphotyrosyl IRP was converted to the monophosphotyrosyl IRP product. The reaction was stopped with trifluoroacetic acid and the phosphopeptides were separated by HPLC. The peptide peaks corresponding to the diphosphotyrosyl peptide were collected and the phosphotyrosine content at each of the three positions was determined by sequencing as described previously (Ramachandran *et al.*, 1992). The mean ± SEM of four experiments is presented.

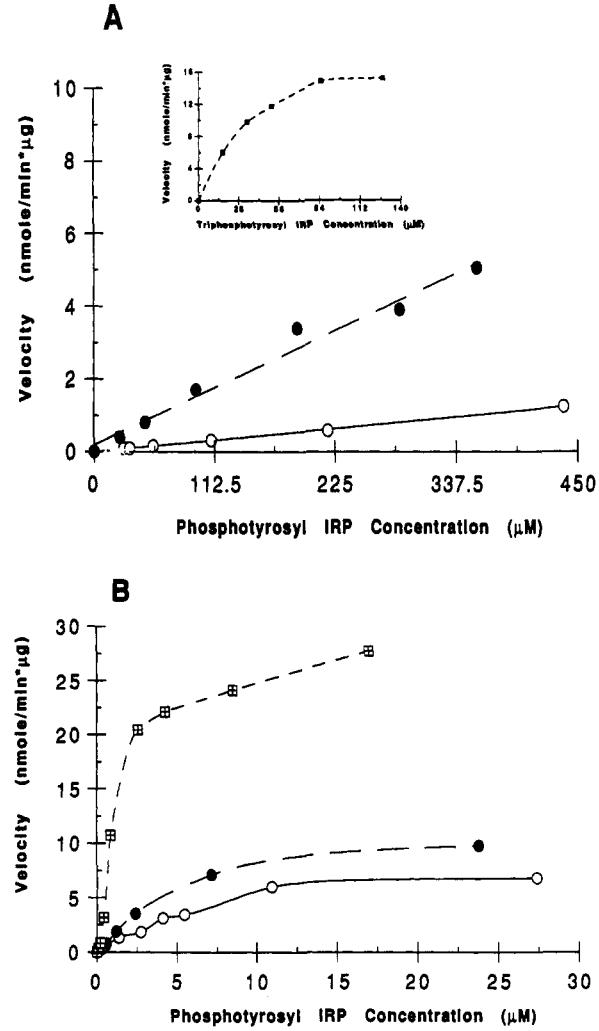

FIGURE 3: Substrate vs velocity curve for the dephosphorylation of phosphotyrosyl IRPs by PTP1C and ΔPTP1C. The initial rate of dephosphorylation of 10-monophosphotyrosyl IRP (○), 5,9-diphosphotyrosyl IRP(●), and triphosphotyrosyl IRP (⊞) were determined with PTP1C (panel A) and ΔPTP1C (panel B) as described in the experimental section. The reaction rates were linear with time and enzyme concentration. Under the conditions used only one phosphotyrosyl residue was dephosphorylated from di- and triphosphotyrosyl IRPs. The data from a typical experiment are presented.

imately 22-fold higher than the K_m for ΔPTP1C using the triphosphotryosyl IRP (Figure 4). Furthermore, the k_{cat}/K_m ratio of PTP1C is 6.55×10^5 M^{-1} s^{-1}, approximately 23-fold lower than that of ΔPTP1C. This shows that the SH2 domains of PTP1C have an inhibitory effect on the phosphatase activity of the enzyme. To address this further we expressed PTP1C without its N-terminal SH2 domain (ΔNPTP1C) and performed kinetic studies with the purified enzyme. The K_m value of ΔNPTP1c was almost identical to that of the fully truncated enzyme when triphosphotyrosyl IRP was used (Figure 4). In addition, the K_m values obtained for ΔNPTP1C using the 10-monophosphotyrosyl IRP (data not shown) were similar to those determined for ΔPTP1C. This suggests that the N-terminal SH2 domains alone can account for the inhibition of the activity of PTP1C. The pH curves generated for all three constructs resulted in a pH optimum of 5.5 (data not shown) and therefore cannot account for the observed differences in K_m's.

DISCUSSION

The role of SH2 domains, thus far, has been restricted to the binding of phosphorylated tyrosine residues of unique sequences of autophosphorylated growth factor receptors and signal transduction molecules (Cantley *et al.*, 1991; Koch

Table II: Summary of the Kinetic Constants for the Dephosphorylation of Phosphotyrosyl IRPs by ΔPTP1C[a]

substrate	K_m (μM)	V_{max} (nmol min^{-1} μg^{-1})	k_{cat}/K_m (s^{-1} M^{-1}) × 10^6
tri-IRP	1.56 ± 0.43	30.8 ± 4.59	15.03
5,9-IRP	7.17 ± 0.78	15.2 ± 1.08	1.61
5,10-IRP	7.72 ± 1.3	20.9 ± 1.34	2.06
9,10-IRP	4.31 ± 0.76	10.6 ± 2.08	1.88
5-IRP	4.67 ± 2.2	6.5 ± 0.50	1.06
9-IRP	7.74 ± 2.1	18.7 ± 3.83	1.83
10-IRP	10.23 ± 1.0	9.3 ± 2.48	0.69

[a] The kinetic constants for the dephosphorylation of phosphotyrosyl IRP were determined from double-reciprocal plots of experiments carried out similar to that shown in Figure 3. The mean ± SEM for the K_m, V_{max}, and k_{cat}/K_m are shown.

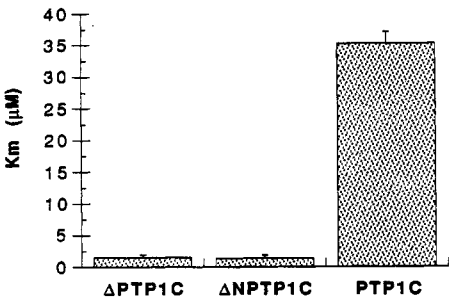

FIGURE 4: Comparison of the K_m values for PTP1C, ΔPTP1C, and ΔNPTP1C. The K_m values for the triphosphotyrosyl IRP were determined as in Figure 3.

et al., 1991; Pawson & Gish, 1992; Waksman *et al.*, 1993). The phosphotyrosyl protein bindng by these domains generates a signaling complex allowing signals to be transduced through a variety of regulatory pathways in response to cellular stimulation, resulting in a physiological response (Carpenter *et al.*, 1993; Skolnik *et al.*, 1993). One particular example of an SH2-containing effector molecule is PTP1C, the first SH2 domain-containing PTP to be identified. PTP1C must, by definition, possess dual phosphotyrosyl binding domain motifs, one with cataltyic activity and one potentially without. Our initial studies using PTP1C generated an interesting observation, namely, that the full-length PTP1C had a lower enzymatic activity than its SH2 domain truncated counterpart. Consequently, this study was set up to address whether the observed differences in activity were due to differing affinities of the PTPs for a substrate. In addition, we investigated whether SH2 domains played any regulatory role in modulating the activity of PTP1C.

We have shown that Michaelis–Menten kinetics were observed for all seven phosphotyrosyl IRPs using ΔPTP1C. Furthermore, ΔPTP1C had the highest affinity (approximately 3–4-fold) and achieved a greater velocity (about 1.5–4-fold) using the triphosphotyrosyl IRP than any of the other mono- or diphosphorylated IRPs. In addition, ΔNPTP1C also had an 8-fold higher affinty for the triphosphotyrosyl IRP than for the 10-monophosphotyrosyl IRP (data not shown). Furthermore, the catalytic efficiency ratio (k_{cat}/K_m) for the triphosphotyrosyl IRP for ΔPTP1C was on average 8-fold higher than for any of the other IRP substrates. For PTP1C, maximal velocity was achieved at approximately 80 μM using the triphosphotyrosyl, IRP; however, maximum velocities for the mono- and diphosphotyrosyl IRPs were still not attained even at substrate concentrations of greater than 800 μM. In parallel with the observations for ΔPTP1C, PTP1C also had the greatest affinity for the triphosphorylated IRP. It is important to note that the substrate concentrations were expressed as moles of IRP. Expressing the concentration of IRP as molar phosphotyrosine would minimize the K_m differences observed between the triphosphotyrosyl IRP and all other phosphotyrosyl IRPs. It will not, however, affect the differences observed for V_{max} and will reduce the magnitude of change in the cataltyic efficiency ratio (k_{cat}/K_m) from 8 to 5-fold for the triphoshpotyrosyl IRP relative to the mono- and diphosphotyrosyl IRPs. Interestingly, the V_{max} values obtained for ΔPTP1C and PTP1C for phosphotyrosyl IPRs fall within the range of values published for LAR and CD45 using phosphotyrosyl IRPs (Tonks *et al.*, 1990; Hyeongjin *et al.*, 1992; Lee *et al.*, 1992). In addition, the K_m values found here for PTP1C were as low as the lowest reported K_m values and the k_{cat}/K_m values were at least 5-fold higher than the highest reported k_{cat}/K_m values for a number of different PTPs with various protein and peptide substrates (Tonks *et al.*, 1988, 1990; Daum *et al.*, 1991; Hyeongjin *et al.*, 1992; Lee *et al.*, 1992; Wang & Pallen, 1992; Zhang *et al.*, 1992).

A comparison of the K_m and k_{cat}/K_m kinetic constants for both PTP1C and ΔPTP1C for the triphosphotyrosyl IRP indicate a 22-fold decrease in K_m and 23-fold increase in k_{cat}/K_m upon truncation of both SH2 domains. There was also an apparent 1.5-fold increase in V_{max} for ΔPTP1C over PTP1C; however, this difference is questionable since the enzyme constructs may possess differing stabilities. Consequently, the concentration of enzyme in the assays may not be a true reflection of the active enzyme present, thereby affecting the observed maximal velocity for the enzyme. On the other hand, K_m values are independent of active enzyme concentrations and can be appropriately compared. Since the truncation of both SH2 domains caused a dramatic change in the enzymes affinity for phosphotyrosyl IRPs, we decided to perform the same kinetic studies on the N-terminal truncated SH2 domain PTP1C. The K_m for ΔNPTP1C for triphosphotyrosyl IRP was almost identical to the K_m value obtained for ΔPTP1C and was approximately 25-fold lower than the K_m value for PTP1C. This suggests that the N-terminal SH2 domain alone can exert an inhibitory effect on the phosphatase activity of PTP1C.

While this work was being completed, Pei *et al.* (1993) overexpressed and purified PTP1C and ΔPTP1C in *E. coli*. They observed a 4–6-fold increase in K_m values for PTP1C when compared to ΔPTP1C using pNPP as a substrate; however, they were not able to obtain saturation kinetics using a variety of phosphotyrosyl pepetide substrates, including IRPs.

There are several possible explanations for the mechanism of inhibition of the activity of PTP1C by its SH2 domains. Since truncation of the SH2 domain of PTP1C increases the affinity of the enzyme for substrates and the magnitude of the increase depends on the substrate [4–6-fold for pNPP (Pei *et al.*, 1993), 22-fold for triphosphotyrosyl IRP, and at least 80-fold for mono- and diphosphotyrosyl IRPs], it is unlikely that the SH2 domain in PTP1C is competing against the substrate for the active site of the enzyme. The SH2 domain may, however, be binding to an allosteric autoinhibitory site on PTP1C. A recent study by Zhao *et al.* (1993) lends support to the presence of an autoinhibitory site in PTP1C. Removal of the 41 amino acid C-terminal region of PTP1C increased the activity of the enzyme. In concert with our ΔNPTP1C kinetic studies, this suggests that the N-terminal SH2 domain of PTP1C may be interacting with its C-terminal region, causing a reduction in activity. Another SH2 domain mediated regulatory phenomenon is observed in the *src* family of tyrosine kinases, which possess an SH2 domain on the same polypeptide chain as the catalytic and autoinhibitory sites (Koch *et al.*, 1991). The C-terminal phosphotyrosyl residue is thought to

interact with the SH2 domain, causing a repression of kinase activity. In analogous fashion, tyrosyl phosphorylation of PTP1C has been demonstrated to occur within the cell (Yeung *et al.*, 1992); therefore, it may also be possible that the SH2 domain of PTP1C binds to a phosphotyroysl residue, resulting in autoinhibition. This scenario seems unlikely, however, since phosphotyrosine was not detected by immunoblotting the PTP1Cs with phosphotyrosine antibody and *E. coli* proteins are not known to contain any protein tyrosine kinases.

Another possibility may be that PTP1C inhibition was due to the SH2 domain binding the phosphotryosyl IRP substrates. This also seems unlikely since the concentration of phosphatase in the assay is in the subnanomolar range whereas that of phosphotyrosyl IRPs is in the micromolar range. In addition PTP1C and ΔPTP1C had the highest affinity for the triphosphotyrosyl IRP substrate over the other phosphotyrosyl IRPs. Furthermore, no selectivity for any of the three phosphotyrosyl positions was observed using triphosphotyrosyl IRP.

In conclusion, our results suggest that the SH2 domains of PTP1C may regulate enzymatic activity via a mechanism other than phosphotyrosyl binding. It would be of interest to map the regions responsible for inhibition of PTP1C and determine whether the regulation of other SH2 domain-containing proteins also occurs independently of phosphotyrosine binding.

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