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Purification and Characterization of a Protein Tyrosine Phosphatase Containing SH2 Domains*

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A protein tyrosine phosphatase (PTP) containing two SH2 domains (PTP1C) was purified to near homogeneity from an adenovirus expression system by a two-step chromatographic procedure with a yield of 67%. The purified enzyme behaves as a monomer of 68 kDa on gel filtration and is totally specific for phosphotyrosyl residues. Its optimal pH is around neutrality for protein substrates such as reduced, carboxamidomethylated, maleylated (RCM)-lysozyme and myelin basic protein but below 5 for low molecular weight compounds such as *para*-nitrophenyl phosphate (*p*-NPP) and phosphotyrosine. Furthermore, with the protein substrates, it displays an activity less than 1% of that obtained with other known PTPs but comparable activities toward *p*-NPP and phosphotyrosine. Its responsiveness toward the usual PTP activators (*e.g.* spermine) or inhibitors (*e.g.* vanadate, molybdate, heparin, or Zn^{2+}) varied considerably with the nature of the substrates involved. Limited digestion with trypsin caused the cleavage of a C-terminal segment of the enzyme, giving rise to a 63-kDa fragment; this cleavage resulted in an approximately 20- and 10-fold activation of the enzyme toward RCM-lysozyme and myelin basic protein, respectively.

Protein tyrosine phosphorylation regulated by the interplay of tyrosine kinases and phosphatases (PTPs),[1] plays a crucial role in signal transduction. While the structure, function, and regulation of protein tyrosine phosphatase have been rather well established (1, 2), those of the PTPs are still lagging behind (for review, see Refs. 3–5). Since the isolation of PTP1B from human placenta (6), more than a dozen intracellular and receptor-like PTPs have been identified. Among these is the novel non-transmembrane enzyme (referred to as PTP1C (7), SH-PTP1 (8), HCP (9), SHP (10), and PTPN6 (11)) containing two SH2 domains upstream from the conserved catalytic core. It is predominantly expressed in hematopoietic cells; its gene is located in the p12-p13 region of human chromosome 12, which is frequently involved in translocations or deletions in acute leukemia (9, 11).

SH2 domains are found in several types of signaling proteins, including a number of intracellular tyrosine kinases. They bind to the sites of autophosphorylation of tyrosine kinase receptors and to other tyrosine-phosphorylated proteins thereby facilitating enzyme-substrate interactions (12). The finding of a PTP with SH2 domains that, presumably, would target this enzyme toward specific substrates is of distinct physiological interest. This article describes the purification and some enzymatic properties of a recombinant PTP1C expressed in 293 cells, using an adenovirus vector.

EXPERIMENTAL PROCEDURES

Materials—*para*-Nitrophenyl phosphate (*p*-NPP), phosphotyrosine, trypsin, and soybean trypsin inhibitor were from Sigma. [γ-^{32}P] ATP and ^{125}I-Protein A were purchased from Amersham Corp. Dulbecco's modified Eagle's medium with high glucose, calf serum albumin, and penicillin/streptomycin antibiotic mixture were from JRH Biosciences. Human 293 cells were obtained from the American Type Culture Collection.

Purified rat brain protein kinase C was a gift from Dr. Sonia Anderson of the Oregon State University. The intracellular domain of the EGF receptor (sEGFR) was purified from a baculovirus expression system (13) kindly provided by Dr. Joseph Schlessinger of New York University. Phosphorylase and phosphorylase kinase were obtained from rabbit skeletal muscle following standard procedures (14, 15). Lipocortin I was purified from bovine lung (16). Tyrosine-phosphorylated RCM-lysozyme and MBP were prepared as previously described (17).

Recombinant Adenovirus and Cell Culture—The coding region of PTP1C was cloned into an adenovirus vector pAdBM-5 which was kindly provided by Dr. Bernerd Massie. The resulting plasmid, pAdBM-2p3, was co-transfected with Ad5/ΔE1AΔE3 genome DNA into 293 cells to generate the recombinant adenovirus, as will be reported in detail elsewhere. The 293 cells were grown in Dulbecco's modified Eagle's medium with high glucose containing 10% calf serum and 100 units/ml each of penicillin and streptomycin. After growing to 50–70% confluency, the cells were infected with the recombinant virus and cultured for another 48 h.

Purification of PTP1C—Virus-infected cells were harvested by centrifugation at 5000 × *g* for 5 min and then washed with phosphate-buffered saline. The cells were broken up with a Dounce glass homogenizer in a buffer containing 25 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, 2 mM EDTA, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 1 μM pepstatin A, and 27 milli-trypsin inhibitory units/ml aprotinin. After centrifugation at 100,000 × *g* for 30 min, the clear supernatant was loaded onto a fast flow Q-Sepharose column (Pharmacia LBK Biotechnology Inc.) equilibrated with buffer A (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 2 mM β-mercaptoethanol). The column was washed with the same buffer and the enzyme eluted with a linear salt gradient of 0–0.5 M NaCl. Fractions containing the PTP activity were combined, concentrated with an Amicon YM10 filter, and then applied to a fast protein liquid chromatography Superose 12 column (Pharmacia). Elution was carried out with buffer A containing 0.1 M NaCl. The active fractions were collected and dialyzed overnight against buffer A containing 50% glycerol and stored at −70 °C.

PTP Assays—Assays with RCM-lysozyme and MBP as substrates

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[1] The abbreviations used are: PTP, protein tyrosine phosphatase; *p*-NPP, *para*-nitrophenyl phosphate; RCM-lysozyme, reduced-carboxamidomethylated-maleylated lysozyme; MBP, myelin basic protein; sEGFR, intracellular domain of epidermal growth factor receptor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

were performed in 25 mM imidazole-HCl, pH 7.0, 1.0 mM EDTA, 1.0 mM dithiothreitol, and 1.0 mg/ml bovine serum albumin, for 5 min at 30 °C (17). The dephosphorylation of *p*-NPP and phosphotyrosine was carried out usually for 4 min at 22 °C in 25 mM sodium acetate, pH 5.0, 20% glycerol, 1.0 mM dithiothreitol, and 1.0 mM EDTA. The *para*-nitrophenol produced was determined by measuring the absorbance at 410 nm in 0.2 M NaOH (18). Activity assays with phosphotyrosine were performed spectrophotometrically as described (19). Phosphatase activity was measured with *p*-NPP throughout the purification. One unit of activity is defined as 1 nmol of phosphate released per min.

Substrate Specificity—The following ^{32}P-labeled substrates were used: glycogen phosphorylase *a* (20), autophosphorylated sEGFR (13), lipocortin I phosphorylated with sEGFR in a similar manner as described for the intact EGF receptor (21), and lipocortin I phosphorylated with protein kinase C (16). The dephosphorylation reactions were performed with 80 μg/ml PTP1C at 30 °C, pH 7.0, for 30 min. The extent of the reaction was analyzed by SDS-PAGE followed by autoradiography and direct measurement of the radioactivity of gel slices corresponding to individual proteins.

Limited Proteolysis—Tryptic digestion of PTP1C (0.4 mg/ml) was performed with 8 μg/ml trypsin at 22 °C for 5 min in a buffer containing 50 mM Tris-HCl, pH 7.5, and 1.0 mM dithiothreitol, and stopped by addition of soybean trypsin inhibitor to a final concentration of 40 μg/ml. For the controls, the inhibitor was added before the protease. The course of the reaction was followed by activity measurements and by SDS-PAGE. In order to identify the cleavage sites, the digest was fractionated on an Applied Biosystem Aquapore RP-300 C8 mini-column (100 × 21-mm) and the fractions obtained were analyzed either with a PE Sciex API III Biomolecular Mass Analyzer or with an Applied Biosystem gas-phase sequenator.

SDS Gel and Western Blotting—SDS-PAGE was run with linear gradient (9–19%) of polyacrylamide on a minigel system according to Laemmli (22) and stained with Coomassie Brilliant Blue R-250. For Western blotting, rabbit polyclonal antibodies raised against either full-length PTP1C purified from an *Escherichia coli* expression system (7) or a synthetic peptide corresponding to the region separating the SH2 from the catalytic domain (Leu^{211}-Asn^{221}) were used as primary antibodies. Detection was carried out with ^{125}I-Protein A. Protein concentration was determined by the Bradford method (23), employing bovine serum albumin as standard.

RESULTS

Purification of PTP1C—Fig. 1*A* illustrates the elution profile of the enzyme from fast flow Q-Sepharose ion-exchange chromatography and Fig. 1*B* from Superose 12 gel filtration. Only one peak of activity in the cytosolic extract was observed because of the high level of expression of the enzyme and the fact that other endogenous PTPs (such as PTP1B or T cell PTP) would remain in the particulate fraction. Table I summarizes the purification. A 20-fold enrichment was achieved with a yield of 67%. Usually, more than 100 μg of enzyme could be obtained from a 150-mm plate of infected cells.

The homogeneity of the material obtained was assessed by SDS-PAGE (Fig. 2*A*) and its molecular weight by gel filtration (*inset* of Fig. 1*B*). The molecular weight of 68,000 obtained from gel filtration following a linear regression analysis agrees with the values obtained by SDS-PAGE (68,000) and calculated from the primary sequence (67,720), indicating that PTP1C behaves as a monomer under these conditions. Antibodies raised against full-length PTP1C and the synthetic peptide recognized the purified recombinant enzyme as well as the enzyme present in crude extract, with the former antibody showing higher reactivity (Fig. 2*B*). Crude extract of uninfected 293 cells or control cells infected with wild type adenovirus showed no immunological cross-reactivity (data not shown).

pH Optimum—The pH dependence of PTPs is known to vary considerably with the nature of the substrates employed (18, 19, 24). This also applies to PTP1C as illustrated in Fig. 3. With *p*-NPP and phosphotyrosine, sharp pH optima of 4.6 and 4.9 were observed, respectively, while with RCM-lysozyme and MBP, the pH optima were just below neutrality.

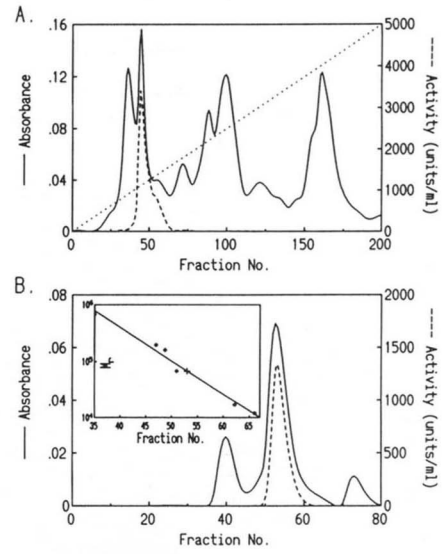

FIG. 1. **Purification of PTP1C by anion exchange chromatography on a fast flow Q-Sepharose column (*A*) and gel filtration on a Superose 12 column (*B*).** ——, absorbance at 280 nm; (- - - -), PTP activity; (· · · ·), 0–0.5 M NaCl gradient. *Inset* in *B* represents the calibration of the Superose 12 column with standard makers (thyroglobulin, 670,000; phosphorylase *b*, 188,000; bovine IG-G, 158,000; bovine serum albumin, 68,000; myoglobin, 17,000; cytochrome *c*, 12,000). The position of PTP1C is indicated by a *plus*, +.

TABLE I
Summary of PTP1C purification

Steps	Volume	Protein	Activity	Specific activity	-Fold	Yield
	ml	*mg/ml*	*units/ml*	*units/mg*		%
Crude	12	1.70	2,600	15,30	1	100
Q-Sepharose	1.1	1.24	22,700	18,300	12	80
Superose 12	0.8	0.87	26,200	30,100	20	67

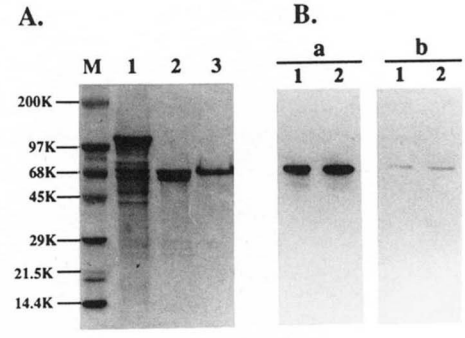

FIG. 2. *A*, SDS-PAGE of samples from PTP1C purification. *Lane M*, protein markers; *1*, crude extract; *2*, fast flow Q-Sepharose column collection; *3*, Superose 12 column collection. *B*, Western blot. *Lane 1*, crude extract (1.0 μg); *lane 2*, purified PTP1C (0.1 μg). Antibody: *a*, anti-full length PTP1C; *b*, antisynthetic peptide. Molecular weight of the standard protein markers for both gels are shown on the *left*.

Kinetics of PTP1C—With all four substrates tested, the enzyme obeyed Michaelis-Menten kinetics; the kinetic constants are listed in Table II. With the two protein substrates, PTP1C showed V_{max} 2 to 3 orders of magnitude lower than obtained with other PTPs such as human placenta PTP1B (18), T-cell PTP, the C terminus truncated form of T-cell PTP (25), receptor PTPα (26), and CD45 (27). MBP was a particularly poor substrate. By contrast, with *p*-NPP and phosphotyrosine as substrates, both sets of kinetic constants were comparable to those reported for several other PTPs (19, 24). Although the K_m for *p*-NPP was in the millimolar

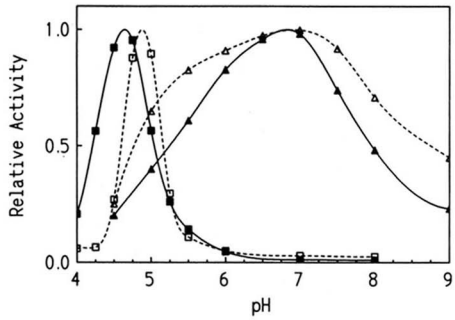

FIG. 3. **pH dependence of PTP1C.** Substrates used were 10 mM *p*-NPP (■), 2 mM phosphotyrosine (□), 3 μM RCM-lysozyme (▲), and 10 μM MBP (△).

TABLE II
Kinetic constants for PTP1C

Substrates	V_{max} (units/mg)	K_m
p-NPP (pH 5.0)	33,000	1.5 mM
Phosphotyrosine (pH 5.0)	8,400	2.0 mM
RCM-lysozyme (pH 7.0)[a]	120	1.9 μM
MBP (pH 7.0)	58	96 μM

[a] After trypsinization, V_{max} of 2,100 units/mg and K_m of 0.9 μM was obtained.

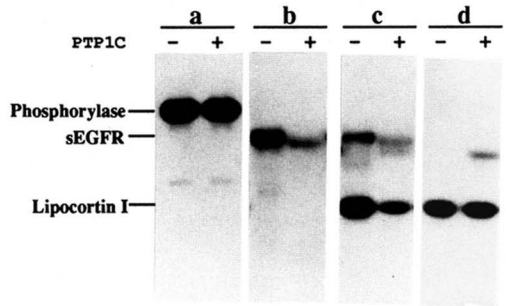

FIG. 4. **Substrate specificity of PTP1C was accessed on glycogen phosphorylase *a* (*a*), sEGFR (*b*), lipocortin I phosphorylated with sEGFR (*c*), or with protein kinase C (*d*).** The minor band observed in the *plus* (+) lane of *panel d* is due to a phosphorylation of added PTP1C by protein kinase C. The stoichiometry of the reaction is less than 0.05 mol of phosphate incorporated per mol of enzyme.

range, its V_{max} was 33,000 nmol/min/mg, corresponding to a turnover number of 37 s^{-1}.

Substrate Specificity—As shown in Fig. 4, glycogen phosphorylase *a*, a classical substrate for Ser/Thr protein phosphatases, was untouched by PTP1C, as was lipocortin I phosphorylated on threonine by protein kinase C. By contrast, autophosphorylated sEGFR and lipocortin I phosphorylated on tyrosine by the sEGFR kinase showed a modest degree of dephosphorylation corresponding to a specific activity of around 50 units/mg. These data indicate that PTP1C behaves as a typical member of the PTP family, displaying almost exclusive specificity for phosphotyrosyl residues on proteins.

Effectors of PTPs—Because of the difference in behavior displayed by PTPs when their activity is measured in the presence of different substrates (18, 19, 24–27), these experiments were carried out with a low molecular weight substrate (*p*-NPP) and two protein substrates, namely, the negatively charged RCM-lysozyme and positively charged MBP. Furthermore, all assays were performed in the presence of 1.0 mM EDTA, known to be an activator of placenta PTP1B (18), even though EDTA by itself had little effect on the activity of PTP1C. The data are presented in Table III. Unexpectedly, sodium vanadate and molybdate, two notorious

TABLE III
Effectors of PTP1C
The data are expressed as relative activity

Effectors		*p*-NPP (10 mM)	RCM-lysozyme (3 μM)	MBP (10 μM)
Control (1 mM EDTA)		1.00	1.00	1.00
Na_3VO_4	0.1 mM	0.03	1.34	1.37
	1.0 mM	0.01	0.57[a]	2.05[a]
$(NH_4)_6Mo_7O_{24}$	10 μM	0.78	1.0	0.86
	100 μM	0.15	1.12[a]	0.33[a]
Heparin	0.2 μg/ml	0.28	0.30	1.79
	2.0 μg/ml	0.01	0.00	3.11
NaF	50 mM	0.93	2.00	0.58
Spermine	0.1 mM	1.04	5.63	0.97
	1.0 mM	1.17	9.33	0.85
$ZnCl_2$	2.0 mM	0.47	0.03	0.08
$MgCl_2$	2.0 mM	1.31	1.31	0.90
	6.0 mM	1.47	3.10	0.68
$MnCl_2$	2.0 mM	1.38	1.66	0.66
	6.0 mM	1.64	3.0	0.34

[a] After trypsin activation, the enzyme is inhibited 100% by 1 mM vanadate and 90% by 0.1 mM molybdate toward both protein substrates.

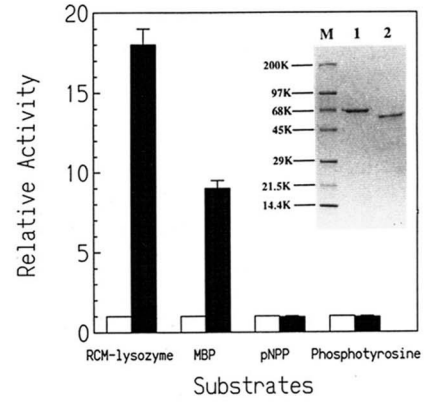

FIG. 5. **Limited proteolysis of PTP1C.** *Open bars* and *solid bars* represent relative activities of control and trypsinized PTP1C towards each individual substrate, respectively. *Inset*: SDS-PAGE of PTP1C before (*lane 1*) and after (*lane 2*) tryptic treatment.

inhibitors of PTPs, had little effect on the activity of the enzyme towards RCM-lysozyme and MBP, although they almost totally abolished the activity towards *p*-NPP. Heparin acted as a potent inhibitor in the presence of *p*-NPP and the acidic protein substrate RCM-lysozyme but not with MBP. By contrast, spermine (and, to a lesser extent, Mg^{2+} and Mn^{2+}) increased by nearly 10-fold the activity of the enzyme in the presence of the negatively charged lysozyme derivative but was essentially without effect on the positively charged MBP. Zinc ions were strongly inhibitory towards the two protein substrates but had much less effect in the presence of *p*-NPP.

Limited Proteolysis of PTP1C—The very low activity displayed by PTP1C towards protein substrates suggested that the enzyme might be repressed by structural motifs outside the catalytic domain. As shown in Fig. 5, limited digestion of PTP1C with trypsin increased its activity approximately 20- and 10-fold towards RCM-lysozyme and MBP, respectively, but had little effect on the activity toward *p*-NPP and phosphotyrosine. The activation reaction was rapid; enzyme activity reached a maximum within 5 min and remained at that level for another 30 min, indicating that the core of the enzyme was relatively resistant to further proteolysis. During this brief treatment, the enzyme was cleaved into a major fragment of about 63 kDa as shown by SDS-PAGE (*inset* of

Fig. 5). The profile of the digest following high performance liquid chromatography is illustrated in Fig. 6; the dominant fraction (peak 3) eluting at 47% acetonitrile corresponded to the 63-kDa band on the SDS-PAGE. No N terminus could be detected on a gas-phase sequenator, suggesting that it arose from the amino end of the molecule which has a blocked N terminus. This was confirmed by further sequencing data obtained after CNBr treatment of the sample (28) and mass spectrometric analysis of the smaller fragments generated. Material eluting under peak 1 (6% acetonitrile) and peak 2 (14% acetonitrile) had molecular masses of 1561.1 and 1915.7 Da, respectively. The former could correspond to either of two peptides of identical mass (Q^{582}-K^{595} or S^{584}-K^{597}) and the latter to peptide T^{557}-K^{572} (Fig. 7). Materials in other small peaks whose masses did not match possible tryptic fragments of PTP1C probably arose from trypsin, trypsin inhibitor, or impurities. On the basis of these data, we conclude that limited tryptic digestion of PTP1C removed 41 amino acid residues (T^{557}-K^{597}) from the C-terminal portion of the enzyme. Limited proteolysis of PTP1C with *Staphycoccus aureus* strain V8 protease, which is specific for acidic amino acid side chains also yielded a major fragment of about 63 kDa and had similar effects on the activity of the enzyme (data not shown).

DISCUSSION

Recombinant PTP1C purified from an adenovirus expression system shares many features with other members of the PTP family, including strict specificity toward phosphotyrosyl residues in proteins and high turnover numbers in the presence of low molecular weight substrates such as *p*-NPP and phosphotyrosine. By contrast, it displays activities 2–3 orders of magnitude lower than the other PTPs in the presence of two commonly used protein substrates, namely, RCM-lysozyme and MBP. The possibility that this low level of activity is due to an autoinhibitory reaction by certain segments of the enzyme itself is supported by the observation that limited proteolysis of PTP1C results in a considerable increase in activity. Table III would suggest that PTP1C is rather insensitive to the action of vanadate and molybdate when assayed in the presence of RCM-lysozyme and MBP.

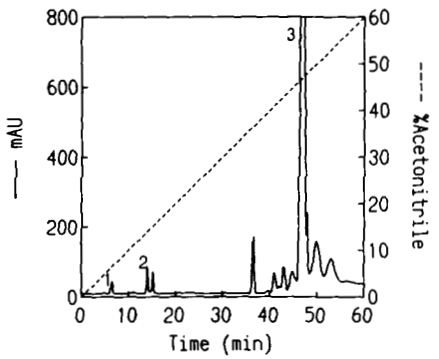

FIG. 6. **Fractionation of the trypsinized product of PTP1C on a reverse-phase high performance liquid chromatography C8 column.**

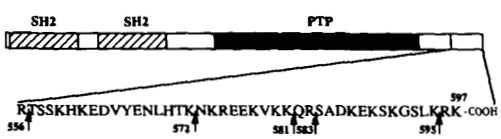

FIG. 7. **C terminus sequence of PTP1C.** *Dashed bars* represent the SH2 domains; *solid bar*, the catalytic domain. The assumed tryptic cleavage sites are indicated by *arrows*.

However, the activity of the enzyme toward these protein substrates is very low and, therefore, the cause of this apparent insensitivity is unclear. These salts could have a dual and compensatory effect: on the one hand, classical inhibition as expected from this class of compounds; on the other hand, activation due from some distortion of the molecule that would unmask its catalytic site. In support of this hypothesis is the observation that after trypsin activation the enzyme is strongly inhibited by vanadate and molybdate (see Footnote a of Table III). While physiologically relevant substrates of PTP1C have not been identified as yet, both lipocortin I phosphorylated on tyrosyl residue and the autophosphorylated kinase domain of the EGF receptor were susceptible to the action of this enzyme *in vitro*.

All known tyrosine phosphatases display an optimum pH in the acidic range in the presence of low molecular weight substrates such as *p*-NPP and phosphotyrosine (18, 19, 24). This also applies to PTP1C. Tyrosine phosphatases are typical "SH enzymes" with a highly conserved sulfhydryl residue shown to participate directly in catalysis. First, mutation of this residue results in total loss of enzymatic activity; second, a phosphoenzyme has been captured and shown to have the property of thiophosphate compounds (29, 30); and third, a thiophosphate derivative was shown by ^{31}P NMR to be produced as an intermediate during the course of the reaction catalyzed by a low molecular weight enzyme with PTP activity from bovine heart (31). Therefore, the rate of the PTP-catalyzed reaction could be determined either by the rate of formation or breakdown of this thiophosphorylated intermediate. While the optimal pH for the breakdown was reported to be as low as 3 (29), an acidic pH would also favor the nucleophilic attack leading to the formation of the thioester bond. Of course, with protein substrates, many other factors would enter into consideration such as the charges of the substrates, and how their conformation and interaction with the enzyme would be affected by changes in pH. Nevertheless, a local acidic environment within the catalytic center might enhance the reaction rate.

PTP1C contains an approximately 80-residue long C-terminal segment outside the catalytic domain. Its rapid cleavage by trypsin suggests that it is rather exposed; the 10–20-fold increase in activity toward RCM-lysozyme and MBP that results from the reaction suggests that it plays a regulatory role. An analogous increase in activity of the T-cell PTP toward RCM-lysozyme was observed following truncation of an 11-kDa C-terminal fragment of the enzyme. In fact, many protein kinases exist in a catalytically inactive state resulting from the presence of autoinhibitory domains residing either on separate subunits (as in the cAMP-dependent protein kinase) or within the same subunit, upstream (*e.g.* protein kinase C) or downstream (*e.g.* myosin light chain kinase) from the catalytic domain (32). Distortion of the molecule following binding of a ligand, covalent modification of this domain, or its removal by limited proteolysis, would unmask the catalytic site and activate the enzyme. Perhaps of more relevance might be the activation of C-src by cleavage of the C-terminal residues of the molecule, or by dephosphorylation of Tyr^{527} thought to bind to the SH2 domain and maintain the enzyme in a folded and repressed conformation (12, 33). There appear to be only traces of phosphotyrosine in the purified recombinant PTP1C as judged by Western blot analysis employing antiphosphotyrosine antibodies. Furthermore, no tyrosine phosphorylation could be detected in the C-terminal segment upon trypsinolysis by mass spectrometry. Whether another group could interact with the SH2 domains, or a particular motif within the C-terminal segment could directly inhibit

the enzyme is not known. On the other hand, recent experiments to be reported elsewhere indicate that PTP1C can be phosphorylated on tyrosyl residues by various intracellular or receptor-linked tyrosine kinases *in vitro*, although the significance of this observation is unclear.

While the receptor forms of the PTPs are for the most part located on the cell surface through their transmembrane segments, the intracellular enzymes are thought to be confined to different regions of the cell through their noncatalytic domains. It can be assumed that these regulatory/localization segments will play an essential role in determining the physiological function of the enzyme. For instance, T-cell PTP associates with the particulate fraction (34) and PTP1B is located in the endoplasmic reticulum (35) through their C-terminal hydrophobic domains. The N-terminal portions of PTP1H and PTP-MEG display homology to the cytoskeletal protein band 4.1, ezrin, and talin that participate in the interaction between the cytoskeleton and the plasma membrane (36, 37). PTP-MEG2 has sequence homologies to the retinaldehyde-binding protein and to yeast SEC14p in its N-terminal domain (38). In this sense, the regulatory domains of the intracellular enzymes may serve a function analogous to the regulatory or targeting subunits found in the protein serine/threonine phosphatases (such as the glycogen-binding subunit of protein phosphatase 1) that direct these enzymes toward their specific substrates (39, 40).

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