Acidic Residues Are Involved in Substrate Recognition by Two Soluble Protein Tyrosine Phosphatases, PTP-5 and rrbPTP-1†

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ABSTRACT: The mechanisms for substrate recognition by two cytoplasmic protein tyrosine phosphatases, PTP-5 and rrbPTP-1, were investigated. Phosphorylation sites on tyrosine-phosphorylated casein, a model PTP substrate, were characterized. Two peptides based on casein phosphorylation sites and one peptide based on the tyrosine phosphorylation site of reduced, carboxamidomethylated and maleylated (RCM) lysozyme were tested as PTP substrates. The three peptides were dephosphorylated by PTP-5 and rrbPTP-1 at rates comparable to those of the corresponding sites on the intact proteins. This indicates that peptides based on the two model PTP substrates, casein and RCM-lysozyme, contained all or most of the structural information necessary for PTP-5 and rrbPTP-1 substrate recognition. Structural elements required for substrate recognition by PTP-5 and rrbPTP-1 were also investigated. K_m values for dephosphorylation of three simple aromatic phosphate esters (phosphotyrosine, *p*-nitrophenyl phosphate, and phenyl phosphate) by rrbPTP-1 were about 5000-fold higher than those obtained for the peptide and protein substrates. This indicates that recognition of protein and peptide substrates involves structural elements in addition to the phosphate group and the aromatic tyrosine ring of phosphotyrosine. Analysis of the effects of truncations and Ala for polar substitutions on the reactivity with PTP-5 and rrbPTP-1 of peptides based on casein, RCM-lysozyme, and angiotensin II indicated that Asp or Glu within the first five residues on the N-terminal side of phosphotyrosine increased peptide reactivity with both PTP's. Asn residues were unable or only weakly able to substitute for Asp residues. These results indicate that one or more acidic residues on the N-terminal side of phosphotyrosine enhance peptide reactivity with PTP-5 and rrbPTP-1 in an additive fashion.

Protein tyrosine phosphatases (PTP's)[1] are enzymes that remove phosphate from tyrosine residues of cellular proteins. They belong to a rather large gene family found in eukaryotes from yeast to man, in prokaryotes of the genus *Yersinia*, and in vaccinia virus (Pot & Dixon, 1992; Krueger & Saito, 1992; Fischer et al., 1991). PTP's can be grouped into three classes. The transmembrane class are single-pass transmembrane proteins with one or two intracellular PTP domains and an extracellular domain of variable structure (Pot & Dixon, 1992; Krueger & Saito, 1992; Fischer et al., 1991). The cytoplasmic class lacks the transmembrane and extracellular domains and has only a single PTP domain (Pot & Dixon, 1992; Fischer et al., 1991). The dual-specificity class has the unique property that its members remove phosphate from Ser and/or Thr residues as well as Tyr (Gautier et al., 1991; Pot & Dixon, 1992).

A critical question in understanding PTP function is how do they recognize their substrates? In this paper we have studied the molecular basis for substrate recognition by two cytoplasmic PTP's. PTP-5 is the major soluble PTP activity toward phosphotyrosylcasein in bovine brain (Jones et al., 1989). PTP-1 is an endoplasmic reticulum localized rat brain PTP that is closely related to human placenta PTP 1B (Guan et al., 1990). Although the primary sequence of PTP-5 is not known, it can be distinguished from PTP-1 immunologically and based on sensitivity to inhibition by Zn^{2+} (G. Kordiyak and T. S. Ingebritsen, unpublished results). Rat brain PTP-1, human placenta PTP 1B, and bovine brain PTP-5 are among the best biochemically characterized PTP's. Previous studies have shown that PTP-5 has a very low K_m for casein (130 nM) (Jones et al., 1989) and that a truncated form of human placenta PTP 1B has a very low K_m for reduced, carboxamidomethylated and maleylated (RCM) lysozyme (80 nM) (Tonks et al., 1988). However, there has not been any attempt to identify features of substrate structure which might account for this high reactivity with casein and RCM-lysozyme. We now show that tryptic phosphopeptides derived from casein and RCM-lysozyme mimic the reactivity of the corresponding tyrosine phosphorylation sites on the intact proteins and that acidic residues on the N-terminal side of phosphotyrosine promote substrate reactivity with bovine brain PTP-5 and rrbPTP-1, a truncated, recombinant form of rat brain PTP-1.

MATERIALS AND METHODS

Materials. The C8 (Brownlee Aquapore RP-300, 0.46 × 22 cm) reverse-phase HPLC column was from Rainin. The mono Q (HR 5/5) FPLC column was from Pharmacia. [γ-^{32}P]ATP was purchased from ICN or prepared from ^{32}P-

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[1] Abbreviations: PTP, protein tyrosine phosphatase; rrbPTP-1, a recombinant form of rat brain PTP-1; RCM-lysozyme, reduced, carboxamidomethylated and maleylated lysozyme; EGF, epidermal growth factor; TFA, trifluoroacetic acid; pNPP, *p*-nitrophenyl phosphate; TCA, trichloroacetic acid; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; PMSF, phenylmethanesulfonyl fluoride; TPCK, *N*-tosylphenylalanine chloromethyl ketone.

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labeled inorganic phosphate (ICN) as described in Johnson and Walseth (1979). Trifluoroacetic acid (TFA) and HPLC-grade acetonitrile were from Applied Biosystems, Inc. Bovine casein (a mixture of 70% α_{s1} + α_{s2} and 30% β + κ caseins), TPCK-treated trypsin from bovine pancreas, calf intestinal alkaline phosphatase attached to beaded agarose, angiotensin II derivatives, *p*-nitrophenyl phosphate (pNPP), phenyl phosphate, and free phosphotyrosine were from Sigma. α-Chymotrypsin was from Worthington Biomedical Corp. RCM-lysozyme was prepared by maleylation, reduction of disulfide bonds, and reaction of free sulfhydryl groups with iodoacetamide to exposure tyrosine residues as described in Tonks et al. (1991). Sources of other reagents have been previously described (Jones et al., 1989).

Peptide Synthesis. Lysozyme- and casein-related peptides were synthesized in the Iowa State University Protein Facility by solid-phase methodology on an Applied Biosystems 430A peptide synthesizer using *N*-methylpyrrolidone or dimethylformamide as solvent for the coupling reactions and *tert*-butyloxycarbonyl protecting groups. Peptides were cleaved from the resin, and side chains were deprotected by treatment with anhydrous HF. The peptides obtained in this way were 70–90% pure. Peptides were characterized by amino acid analysis to verify amino acid composition and fast atom bombardment mass spectrometry to verify molecular weight after purification by reverse-phase HPLC (C18 column) using an acetonitrile/0.1% (v/v) TFA gradient (1%/min) at a flow rate of 1 mL/min.

Preparation of ^{32}P-Labeled Substrates. Casein and RCM-lysozyme were phosphorylated by the insulin receptor using reaction conditions previously described for the phosphorylation of casein (Jones et al., 1989; Ingebritsen, 1991). Phosphate incorporation was 1–5 nmol/mg for casein and 4–12 nmol/mg for RCM-lysozyme. When casein and RCM-lysozyme were used as substrates, they were further processed to remove unreacted ATP as previously described (Jones et al., 1989; Ingebritsen, 1991).

Synthetic lysozyme-, casein-, and angiotensin II-related peptides were phosphorylated by the intact heterotetrameric insulin receptor kinase, under the same reaction conditions used for casein and RCM-lysozyme, or by a soluble form of the β subunit (BIRK), using slightly modified conditions in which insulin and the detergent Triton X-100 were omitted from the reaction buffer. Stoichiometries of phosphorylation ranged from 0.02 to 0.85 mol/mol for the various peptides. Lysozyme- and casein-related peptide phosphorylation reactions were stopped with an equal volume of 0.1% (v/v) TFA and purified either by a combination of chromatography on Sep-Pak C18 cartridges to remove unreacted ATP, followed by reverse-phase chromatography (C8 column), or by reverse-phase chromatography (C8 column) alone. Sep-Pak cartridges were washed with 10% (v/v) acetonitrile/0.1% (v/v) TFA to remove unreacted ATP, and phosphopeptides were eluted with 100% (v/v) acetonitrile/0.1% (v/v) TFA. Phosphopeptides were separated from the nonreacted peptides by reverse-phase HPLC (C8 column). Baseline separations of phosphorylated and unphosphorylated peptides were achieved using a linear acetonitrile/0.1% (v/v) TFA gradient (0.2%/min) across the two peptide peaks. Angiotensin II-related peptide phosphorylation reactions were terminated and processed in the same way, but it was not possible to completely separate phosphorylated angiotensin II-related peptides from unreacted peptides by HPLC chromatography (C8 column).

Phosphopeptide 69-3 was generated by chymotrypsin digestion (1:12.5 w/w) of HPLC-pure phosphopeptide 67-2 (see Table III for sequences). The HPLC-pure phosphopeptide was dried using a Speed-Vac, dissolved in 100 μL of 1% (w/v) ammonium bicarbonate, and digested for 3.5 h at 30 °C. The digestion was stopped with 400 μL of 0.1% (v/v) TFA, and the products were separated by reverse-phase HPLC (C8 column) using a linear acetonitrile/0.1% (v/v) TFA gradient (1%/min) at a flow rate of 1 mL/min. A single ^{32}P-labeled digestion product was observed. In order to identify the digestion product, the phosphopeptide was dephosphorylated by incubation with 2 units of rrbPTP-1 for 12 h and subjected to amino acid analysis. The results showed that the digestion product arose due to cleavage by chymotrypsin after the leucine residue rather than after the phosphorylated tyrosine residue.

Peptide Mapping. For casein the phosphorylation reaction was stopped by addition of trichloroacetic acid (TCA) to a final concentration of 20%; the precipitate was washed with 5% TCA and redissolved in 50 mM ammonium bicarbonate, pH 8.5–1.0 mM EDTA–2 M urea (buffer A). TPCK-trypsin (1:40 w/w) was added and the mixture incubated at 30 °C for 16 h. An aliquot of the mixture was adjusted to pH 4 with TFA and subjected to reverse-phase HPLC (C8 column). The column was developed with a linear acetonitrile–0.1% TFA gradient (1%/min) at a flow rate of 1 mL/min. When peptide mapping reactions were run on an analytical scale, additional unlabeled casein was added to the trypsin digestion reaction as a carrier.

Peptide Sequencing. A tryptic digest of phosphorylated casein was applied to a FPLC Mono Q column (Pharmacia) equilibrated with 20 mM Tris-HCl (pH 7.5 at 25 °C), and ^{32}P-labeled peptides were eluted with a linear 0–1.0 M NaCl gradient (30-mL total volume) at a flow rate of 1 mL/min. Two radioactive peptides peaks were obtained eluting at 300 mM NaCl (a mixture of peptides 1, 3, and 4) and 575 mM NaCl (peptide 2) (not shown). Casein peptides 1 and 2 were further purified to homogeneity by subjecting the leading edge of FPLC peak 1 and the entire FPLC peak 2 to reverse-phase HPLC (C8 column, see above). The three peptides were sequenced by stepwise automated Edman degradation after an initial double-coupling protocol using an Applied Biosystems Model 470A gas-phase sequenator equipped with an on-line Model 120A PTH-amino acid analyzer. Casein peptide 2 was also sequenced after dephosphorylation by overnight incubation with 0.5 unit of immobilized calf intestinal alkaline phosphatase.

PTP Assays. PTP assays using protein substrates were carried out using conditions previously described for the assays of PTP-5 (Jones et al., 1989; Ingebritsen, 1991). The same procedure was used for peptide substrates except that [^{32}P]P_i produced during the incubation was separated from unreacted phosphopeptide either by extraction of phosphomolybdate complexes into organic solvent (Shenolikar & Ingebritsen, 1984) or by binding unreacted phosphopeptide to activated charcoal (Streuli et al., 1990). When pNPP was used as substrate, the reaction was terminated by addition of 1 mL of 0.2 M NaOH, and the appearance of the *p*-nitrophenolate anion was monitored by absorbance at 410 nm (Jones et al., 1989; Ingebritsen, 1991). Dephosphorylation of phosphorylated tyrosine and phenyl phosphate was monitored by the appearance of unlabeled inorganic phosphate using a malachite green assay (Van Veldhoven & Mannerts, 1987).

Data Analysis. Apparent K_m and V_m values for the two protein tyrosine phosphatases with the various substrates were determined from initial rate experiments using nonlinear regression analysis (Cleland, 1967). Uncertainty in the kinetic

parameters were estimated in two ways. In the experiments presented in Tables II, III, and V and in part of Table VI, the SE parameter was estimated from the data scatter in the nonlinear least squares fit involving 12 data points. Alternatively, in the experiments presented in Table IV and in part of Table VI, the kinetic analysis was repeated three times, and the uncertainty was estimated by the SEM of the parameter. The SE and SEM values for the parameters were similar. Statistical comparisons were made using the Student t test.

Expression and Purification of rrbPTP-1 from Escherichia coli. The PTP-1 used in this study is a truncated, recombinant form of rat brain PTP-1 (rrbPTP-1) produced by replacing the Lys-323 codon with a stop codon by site-directed mutagenesis (Guan & Dixon, 1991). Two different vectors (pT7-PTPU323 and pKG-PTPU323) were used to express the phosphatase in *E. coli*, and different protocols were used to purify each to homogeneity. rrbPTP-1 expressed from pT7-PTPU323 or pKG-PTPU323 differs in its N-termini because of sequences added from their respective vectors. The authentic N-terminus of rat brain PTP-1 is MEMEKE; the N-terminus from pT7-PTPU323 is MARIRAQ, and that for pKG-PTPU323 is GSPGISGGGGGIRAQ. The two forms of rrbPTP-1 had comparable K_m and k_{cat} values with RCM-lysozyme, peptides 65-1 and 67-2, and p-nitrophenyl phosphate.

The vector pT7-PTPU323 was used to express rrbPTP-1 in *E. coli* behind the bacteriophage T7 promoter (Guan et al., 1990). *E. coli* from 1 L of culture were lysed by French press in 10 mM Tris-HCl, pH 7.4–1 mM EDTA–0.2 mM PMSF–0.05% 2-mercaptoethanol. The lysate was centrifuged at 12000g for 15 min, and the supernatant (extract) was further centrifuged at 100000g for 60 min. The supernatant (cytosol) was applied to a DE-52 column (1.6 × 12 cm) equilibrated in 20 mM Tris-HCl, pH 7.0 at 25 °C–0.1 mM EDTA–0.1 mM PMSF–0.1 mM benzamidine–0.2% 2-mercaptoethanol (buffer A) at a flow rate of 30 mL/h. The column was washed with buffer A until the protein concentration was <0.02 mg/mL (~60 mL). The DE-52 column was eluted with a 300-mL linear gradient (0–500 mM NaCl) in buffer A at a flow rate of 30 mL/h. Then 5-mL fractions were collected; the single peak of PTP activity, eluting between 80 and 120 mM NaCl, was pooled, dialyzed overnight against one change of 10 mM imidazole hydrochloride, pH 7.2–5 mM EDTA–0.5 mM benzamidine–0.5 mM PMSF–0.1% 2-mercaptoethanol (buffer B), and applied to a thiophosphorylated RCM-lysozyme column (1.5 × 15 cm) equilibrated with buffer B at a flow rate of 40 mL/h. The flow-through material was collected and reapplied to the column. The column was washed with four bed volumes of buffer E and eluted with buffer B containing 250 mM NaCl. Fractions containing the PTP activity peak were pooled, dialyzed overnight against buffer B containing 50% glycerol, and stored at –20°C.

Expression and purification of rrbPTP-1 from the vector pKG-PTPU323 was accomplished essentially as described in Guan and Dixon (1991). Briefly, rrbPTP-1 was expressed in *E. coli* as a fusion protein with glutathione S-transferase. The fusion protein was affinity purified directly from bacterial homogenates using glutathione–agarose, and the rrbPTP-1 was liberated from the beads by cleavage of the fusion protein with the specific protease thrombin.

Other Protein Preparations. The insulin receptor kinase was partially purified through the wheat germ agglutinin–Sepharose chromatography step as described in Treadwell et al. (1989). BIRK, a soluble, catalytically active form of the β subunit of the insulin receptor tyrosine kinase, was purified from Sf9 cells through the Sepharose fast Q step as described in Herrera et al. (1988). Bovine brain PTP-5 was partially purified through the phosphocellulose step as described in Jones et al. (1989) and is free of other bovine brain PTP's.

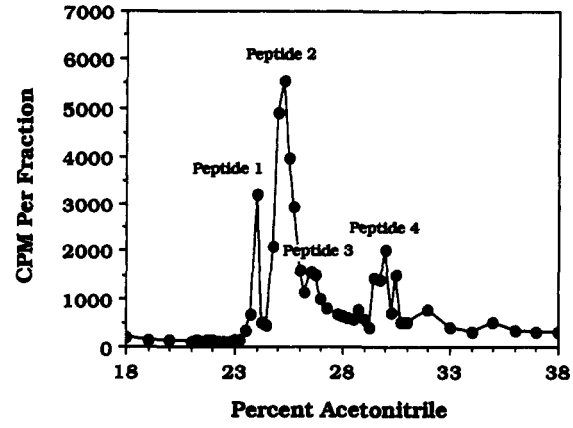


FIGURE 1: Peptide mapping of the tyrosine phosphorylation sites of casein. A reverse-phase HPLC chromatogram of the total tryptic digest is shown. Other conditions are described in Materials and Methods.

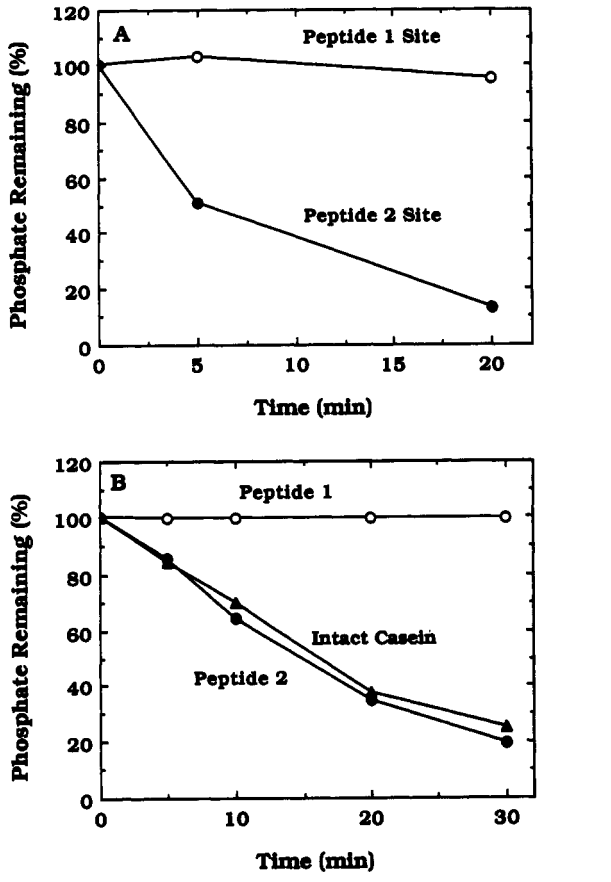
RESULTS AND DISCUSSION

Dephosphorylation by PTP-5 of Individual Tyrosine Phosphorylation Sites on Casein and Tryptic Peptides Derived from Casein. Because of the high reactivity of phosphotyrosylcasein with PTP-5, it was of interest to identify the tyrosine phosphorylation sites on the protein. Tryptic digestion followed by peptide mapping revealed the presence of one major and three minor ^{32}P-labeled peptides (Figure 1). The major peptide (casein peptide 2) and a minor peptide (casein peptide 1) were sequenced (see below), and each contains a single tyrosine phosphorylation site.

Because of the presence of multiple tyrosine phosphorylation site on casein, it was of interest to compare the rates of dephosphorylation of individual sites by PTP-5. The relative rates of dephosphorylation of the sites corresponding to casein peptides 1 and 2 by PTP-5 were determined. Tyrosine-phosphorylated casein was incubated with PTP-5, and at various times aliquots were removed, digested with trypsin and subjected to peptide mapping by reverse-phase HPLC. The amount of phosphate remaining in the casein peptide 1 site and casein peptide 2 site was determined at each time point (Figure 2A). The results show that phosphate was rapidly removed from the casein peptide 2 site whereas the casein peptide 1 site was hardly dephosphorylated at all.

The rates of dephosphorylation of isolated casein peptides 1 and 2 by PTP-5 were also tested. It was found that casein peptide 2 was dephosphorylated by PTP-5 at a rate that was similar to that of intact casein, while casein peptide 1 was not dephosphorylated at an appreciable rate (Figure 2B). Thus the rate of dephosphorylation of the isolated casein peptide 2 closely matched the rate of dephosphorylation of the casein peptide 2 site in the intact protein. Just as striking was the observation that the phosphate on the casein peptide 1 site was not removed at an appreciable rate in either the protein or the isolated peptide.

Sequencing of Casein Peptide 1 and Casein Peptide 2. Casein peptide 2 and casein peptide 1 were purified and sequenced by automated Edman degradation (Table I). The sequences obtained for casein peptides 1 and 2 corresponded to residues 200–205 and 46–55 of α_{s2}-casein, respectively



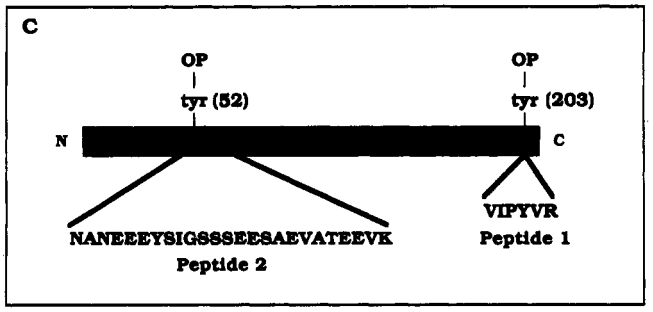


FIGURE 2: Comparison of the rates of dephosphorylation of casein peptides 1 and 2 with the rates of dephosphorylation of the corresponding sites on intact casein. (A) Dephosphorylation of the sites on intact casein corresponding to casein peptide 1 (○) and casein peptide 2 (●). ^{32}P-Labeled casein (50 nM) was incubated with PTP-5 for the indicated times. Dephosphorylation reactions were stopped by addition of TCA (final concentration 25%) and subjected to tryptic digestion. Tryptic peptides were separated by reverse-phase HPLC (C8 column), and ^{32}P radioactivity associated with casein peptides 1 and 2 was quantitated by Cerenkov counting. (B) Dephosphorylation of isolated casein peptide 1 (30 nM) (○), casein peptide 2 (110 nM) (●), and intact casein (100 nM) (▲) by PTP-5. ^{32}P-Labeled casein peptides 1 and 2 were released from intact casein by tryptic digestion and partially purified by reverse-phase HPLC (C8 column). The two peptides as well as intact casein were incubated with PTP-5 for the indicated times and processed to determine the extent of [^{32}P]P_i released during the reaction. Further details are provided by Materials and Methods. (C) Location and sequence surrounding tyrosine phosphorylation sites of α_{s2}-casein.

(Brignon et al., 1977). Since the bovine casein used in our experiments is a mixture of 70% α_{s1} + α_{s2} and 30% β + κ caseins, this indicates that the α_{s2}-casein variant is the major substrate for the insulin receptor kinase.

A gap in the sequence of each peptide was detected at the position expected for phosphotyrosine (Table I). Additionally, the sequence of casein peptide 2 was incomplete relative to

Table I: Sequencing of Casein Peptides 1 and 2[a]

	casein peptide 2			casein peptide 1	
cycle	amino acid	pmol/cycle −AP	pmol/cycle +AP	amino acid	pmol/cycle, −AP
1	N	396		V	373
2	A	447	976	I	379
3	N	267	760	P	334
4	E	277	816	Y	0
5	E	368	496	V	331
6	E	365	736	R	18
7	Y	0	496		
8	S	249	268		
9	I	111	304		
10	G	40	165		
11	S		130		
12	S		122		
13	S		119		
14	E		150		
15	E		184		
16	S		65		
17	A		61		
18	E		113		
19	V		71		
20	A		50		
21	T		14		
22	E		78		
23	E		77		
24	V		40		
25	K		15		

[a] Peptides were sequenced by automated Edman degradation before and after treatment with alkaline phosphatase (AP). Details of peptide isolation and sequencing are given in Materials and Methods. The inability to detect Asn in cycle 1 after treatment of casein peptide 2 with alkaline phosphatase was due to a contaminant that obscured the Asn peak. Quantities sequenced: casein peptide 2 (−AP), 0.65 nmol; casein peptide 2 (+AP), 2.0 nmol; casein peptide 1 (−AP), 0.35 nmol.

that expected for a tryptic peptide, probably due to the presence of phosphate on Ser residues 56, 57, 58, and 61 (Brignon et al., 1977). The gaps in sequence at the positions of phosphotyrosine and phosphoserine were expected because of difficulties in detecting the phenylthiohydantoin derivatives of the phosphoamino acids (Patschinsky et al., 1982; Turck et al., 1991). In order to confirm the presence of phosphotyrosine and to obtain the complete sequence of casein peptide 2, it was treated with alkaline phosphatase to remove phosphate residues and then resequenced (Table I). Following this treatment tyrosine was detected at cycle 7. Since each peptide contained only a single tyrosine residue, the phosphorylation sites of casein peptide 2 and casein peptide 1 can be unambiguously assigned as Tyr-52 and Tyr-203, respectively, of a_{s2}-casein (Figure 2C).

Kinetic Analysis of the Dephosphorylation of Casein Peptide 2 and Lysozyme Peptide 65-1 by PTP-5. The ability of PTP-5 to dephosphorylate peptide substrates was further analyzed by comparing the kinetic constants for dephosphorylation of casein peptide 2 with those for dephosphorylation of intact casein (Table II). The K_m and relative V_m values for dephosphorylation of casein peptide 2 and intact casein were similar, confirming the initial rate results shown in Figure 2B. Kinetic constants for dephosphorylation of casein peptide 1 were not determined since casein peptide 1 was not detectably dephosphorylated by PTP-5 (see above).

RCM-lysozyme is another high-affinity substrate for PTP-5 (Table II), and the major site phosphorylated by a mixture of the epidermal growth factor and insulin receptor kinases is Tyr-53 (Tonks et al., 1991). We have confirmed this for RCM-lysozyme phosphorylated by the insulin receptor kinase alone (results not shown). We synthesized a 16-residue peptide (lysozyme peptide 65-1) containing this phosphorylation site

Table II: Kinetic Constants for Dephosphorylation of Tyrosine Phosphorylated Proteins and Peptides as Well as Simple Aromatic Phosphate Esters by rrbPTP-1 and PTP-5[a]

substrate	rrbPTP-1		PTP-5	
	K_m (μM)	rel V_m (%)	K_m (μM)	rel V_m (%)
casein	0.08	100	0.15	100
casein peptide 2	0.08	73	0.09	106
casein peptide 121	0.20	100	0.64	63
RCM-lysozyme	0.17	128	0.05	15
lysozyme peptide 65-1	1.0	108	2.0	38
lysozyme peptide 67-2	0.6	81	1.6	33
angiotensin II	15.5	166	ND	ND
phosphotyrosine	5100	32	ND	ND
p-nitrophenyl phosphate	4400	52	ND	ND
phenyl phosphate	2000	93	ND	ND

[a] The values presented are from a single experiment. SE estimates for the parameters averaged 12% (range 2–27%) of the parameter for the K_m values and 6% (range 2–19%) of the parameter for the V_m values. For each PTP, V_m values are expressed relative to those obtained using casein as substrate. Relative values are presented because a partially purified PTP-5 preparation was used in these experiments. It should be noted that the V_m (10 μmol·min^{-1}·mg^{-1}) previously obtained for dephosphorylation of phosphotyrosylcasein by a nearly pure PTP-5 preparation (Jones et al., 1989) is similar to the V_m obtained with rrbPTP-1 using this substrate (15 μmol·min^{-1}·mg^{-1}). Further details are given in Materials and Methods.

and tested it as a PTP-5 substrate in comparison with intact RCM-lysozyme (Table II). The peptide was an excellent substrate with a K_m of 2.0 μM although this value was 40-fold greater than that obtained using intact RCM-lysozyme. The relative V_m value for the peptide was 2.5-fold higher than that for intact RCM-lysozyme. These results indicate that casein peptide 2 and lysozyme peptide 65-1 retain most of their activity as PTP-5 substrates compared to the intact proteins from which they were derived.

Dephosphorylation of Casein and Lysozyme Peptides by rrbPTP-1. In order to determine whether high reactivity with casein and lysozyme peptides was a unique feature of PTP-5, a truncated, recombinant form of rat brain PTP-1 (rrbPTP-1) was assayed using casein, casein peptide 2, RCM-lysozyme, and lysozyme peptide as a substrate. Kinetic constants for dephosphorylation of the four substrates are shown in Table II. The K_ms for dephosphorylation of intact casein and casein peptide 2 were comparable whereas the K_m for dephosphorylation of the lysozyme peptide was 6-fold higher than for intact RCM-lysozyme. The V_m values for all four substrates were similar. Casein peptide 1, tested at a single substrate concentration of 100 nM, was dephosphorylated at a very slow rate [(4×10^6)-fold lower than the rate toward casein] (results not shown). These results are similar to those obtained for PTP-5 (see above).

Reactivity of rrbPTP-1 with Aromatic Phosphate Esters. In order to examine the importance of the phosphate group and of the aromatic tyrosine ring in PTP substrate recognition, the reactivities of rrbPTP-1 with phosphotyrosine, *p*-nitrophenyl phosphate, and phenyl phosphate were tested (Table II). The K_m values for the three substrates (2–5 mM) were 4–5 orders of magnitude higher than the K_m values obtained with casein and RCM-lysozyme. In contrast, the V_m values for the three substrates were within a factor of 3 of those obtained with the two protein substrates. This indicates that recognition of protein and peptide substrates involves structural elements in addition to the phosphate group and aromatic tyrosine ring of phosphotyrosine.

Effect of N- and C-Terminal Truncations on the Reactivity of the Lysozyme Peptide with rrbPTP-1 and PTP-5. To further examine the importance of peptide structure in substrate recognition by rrbPTP-1, a series of lysozyme-related peptides were synthesized. Lysozyme was chosen as the model rather than casein because of technical complications with casein peptide 2 synthesis. Casein peptide 2 contained several phosphoserine residues and was 9 amino acids longer than the lysozyme peptide. Each lysozyme related peptide was phosphorylated on tyrosine by the insulin receptor kinase, and the kinetic parameters for dephosphorylation by rrbPTP-1 were examined (Table III).

Deletion of three or five residues from the C-terminus of the lysozyme peptide (peptides 67-2 and 69-3, respectively) slightly improved reactivity by decreasing the K_m for rrbPTP-1. In contrast, deletion of three or six amino acids from the N-terminus of the lysozyme peptide (peptides 66-2 and 66-1, respectively) impaired reactivity by increasing the K_m for rrbPTP-1 by 6- and 24-fold, respectively. The N- or C-terminal deletions did not have any consistent effect on the V_m for the reactions.

The effects of truncations (Table III) on the reactivity on lysozyme-related peptides with PTP-5 were examined. Deletion of three or six residues from the N-terminus of the lysozyme peptide increased the K_m for the reaction with PTP-5 by 4.2- and 31-fold, respectively. Conversely, deletion of three or five residues from the C-terminus of the peptide did not increase the K_m for the reaction with PTP-5. No consistent effects of deletions on the V_ms for the reaction with PTP-5 were observed.

These results indicate that the peptide sequence on the N-terminal side of phosphotyrosine influences peptide reactivity with both rrbPTP-1 and PTP-5 by changing the K_m for the reaction and that three amino acids on the C-terminal side of phosphotyrosine are sufficient to maintain the low K_m with the two PTP's.

Effect of Acidic Residues on Lysozyme Peptide Reactivity with rrbPTP-1. Acidic residues have been implicated as determinants in substrate recognition by protein tyrosine kinases (see below). The effect of the Asp residues at the −1 and −5 positions of the lysozyme peptide sequence was examined by comparing the reactivity of the wild-type lysozyme peptide (67-2) with that of peptide 115 which contains Ala residues at both positions (Table IV). The presence of the two Asp residues decreased the K_m by almost

Table III: Effect of Truncations on the Reactivity of Lysozyme-Related Peptides with rrbPTP-1 and PTP-5[a]

peptide	sequence	rrbPTP-1		PTP-5	
		K_m (μM)	V_m (%)	K_m (μM)	V_m (%)
65-1	NTDGSTDYGILQINSR	1.0 ± 0.1	100 ± 3	2.0 ± 0.3	100 ± 9
66-2	GSTDŸGILQINSR	5.6 ± 0.2[b]	205 ± 3[b]	8.4 ± 0.6[b]	106 ± 6
66-1	DŸGILQINSR	23.8 ± 2.1[b]	98 ± 6	62.5 ± 3.2[b]	75 ± 2[c]
67-2	NTDGSTDŸGILQI	0.6 ± 0.1[c]	75 ± 2[b]	1.6 ± 0.1	86 ± 3
69-3	NTDGSTDŸGIL	0.5 ± 0.1[c]	43 ± 2[b]	1.7 ± 0.1	82 ± 2

[a] The values presented ± SE are from a single experiment. For each PTP, V_m values are expressed relative to lysozyme peptide 65-1. Further details are described in Materials and Methods. [b] $p < 0.001$ vs peptide 65-1. [c] $p < 0.05$ vs peptide 65-1.

Table IV: Effect of Ala for Polar Substitutions on Peptide Reactivity with rrbPTP-1[a]

peptide	sequence	K_m (μM)	rel V_m (%)
67-2	NTDGSTDYGILQI	0.8 ± 0.1[b]	100
77	AAAGSTDYGILQI	2.8 ± 0.2[d]	100 ± 10
112	ATDGSTDYGILQI	1.3 ± 0.5	104 ± 10
68-1	NADGSTDYGILQI	0.9 ± 0.1	113 ± 9
111	NTAGSTDYGILQI	1.4 ± 0.3[b,f]	94 ± 10
76	NTDGAAAYGILQI	2.6 ± 0.2[d]	110 ± 12
68-2	NTDGATDYGILQI	1.1 ± 0.3	115 ± 11
109	NTDGSADYGILQI	0.7 ± 0.2	96 ± 13
110	NTDGSTAYGILQI	2.1 ± 0.3[b,e]	98 ± 5
114	NTNGSTNYGILQI	4.8 ± 0.4[c]	121 ± 11
115	NTAGSTAYGILQI	7.5 ± 0.7	108 ± 11

[a] K_m and V_m values represent the mean ± SEM of three determinations made with separate phosphopeptide preparations. For each preparation V_m values were normalized to the V_m for peptide 67-2. Further details are given in Materials and Methods. [b] $p < 0.005$ vs peptide 115. [c] $p < 0.05$ vs peptide 115. [d] $p < 0.005$ vs peptide 67-2. [e] $p < 0.05$ vs peptide 67-2. [f] $p < 0.05$ vs peptide 77.

Table V: Dephosphorylation by rrbPTP-1 of Peptides Related to Angiotensin II and the Tyr-52 Phosphorylation Site of α_{s2}-Casein[a]

peptide	sequence	K_m (μM)	rel V_m (%)
casein-related peptides			
121	NANEEEYSIG	0.2 ± 0.01	100 ± 3
120	NANAAAYSIG	2.8 ± 0.4[b]	127 ± 12
angiotensin II-related peptides			
AT-1	DRVYIHPF	16 ± 4	100 ± 13
AT-2	SarRVYIHPF	58 ± 7[c]	107 ± 10
AT-3	RVYIHPF	41 ± 2[c]	108 ± 10
AT-4	DRVYVHPF	15 ± 2	89 ± 6
AT-5	NRVYVHPF	43 ± 2	101 ± 4

[a] The values presented ± SE are from a single kinetic experiment. V_m values for casein-related peptides and angiotensin II-related peptides are expressed relative to peptides 121 and AT-1, respectively. Note that peptide AT-1 corresponds to angiotensin II. Other conditions are described in Materials and Methods. [b] $p < 0.001$ vs casein peptide 121. [c] $p < 0.001$ vs peptide AT-1.

an order of magnitude from 7.5 to 0.8 μM without changing the V_m for the reaction. In contrast, substitution of Asn residues at positions −1 and −5 for Ala residues had only a small (less than 2-fold) effect on peptide reactivity (compare peptides 114 and 115). The effect of individual Asp residues was examined by comparing the reactivity of peptides with a single Asp residue at the −1 position (peptide 111) or the −5 position (peptide 110) with the double Ala peptide (peptide 115). The presence of an Asp residue at the −1 position decreased the K_m from 7.5 to 1.4 μM compared with the double Ala peptide whereas the presence of an Asp at the −5 position decreased the K_m to 2.1 μM. These results indicate that Asp residues at the −1 and −5 positions enhance peptide reactivity in an additive fashion by decreasing the K_m for the reaction.

Acidic Residues Also Enhance the Reactivity of Casein Peptide 2 and Angiotensin II with rrbPTP-1. To further investigate the importance of acidic residues N-terminal to the phosphorylated tyrosine residue, we determined the effects of deletions and amino acid substitutions on the kinetics for dephosphorylation of two other rrbPTP-1 peptide substrates. The presence of the Asp residue at position −3 relative to phosphotyrosine in the angiotensin II peptide markedly decreased the K_m for the reaction (16 vs 41–58 μM) compared with peptides containing Sar (*N*-methylglycine) or no residue at this position (compare peptides AT-2 and AT-3 with peptide AT-1 in Table V). The magnitude of the effect was comparable to the effect of a single Asp residue on the reactivity of the lysozyme peptide. The Asp residue had no effect on V_m for the reactions. The presence of Asn at the −3 position

Table VI: Effect of Ala Substitutions on the Dephosphorylation by PTP-5 of Peptides Related to Lysozyme and the Tyr-52 Phosphorylation Site of α_{s2}-Casein[a]

peptide	sequence	K_m (μM)	rel V_m (%)
lysozyme-related peptides			
67-2	NTDGSTDYGILQI	1.8 ± 0.1[b]	100
112	ATDGSTDYGILQI	2.6 ± 0.3	120 ± 30
111	NTAGSTDYGILQI	4.0 ± 0.8[b]	101 ± 7
110	NTDGSTAYGILQI	3.9 ± 1.5[c]	104 ± 42
114	NTNGSTNYGILQI	8.7 ± 0.2[c]	111 ± 10
115	NTAGSTAYGILQI	11.4 ± 0.9	106 ± 8
casein-related peptides			
121	NANEEEYSIG	0.6 ± 0.06	100 ± 4
120	NANAAAYSIG	25 ± 5[d]	233 ± 46[e]

[a] Values for lysozyme-related peptides represent the mean ± SEM of three separate determinations made with separate phosphopeptide preparations. Values ± SE for the two casein-related peptides are from a single kinetic experiment. V_m values for lysozyme-related peptides and casein-related peptides were normalized to peptides 67-2 and 121, respectively. For further details see Materials and Methods. [b] $p < 0.005$ vs lysozyme peptide 115. [c] $p < 0.05$ vs lysozyme peptide 115. [d] $p < 0.001$ vs casein peptide 121. [e] $p < 0.01$ vs casein peptide 121.

did not significantly improve peptide reactivity (compare peptide AT-5 with peptides AT-2 and AT-3). Again the V_m values for the peptides were similar.

The effect of Glu residues on peptide reactivity was examined using a 10-residue peptide based on the sequence surrounding the Tyr-52 phosphorylation site of α_{s2}-casein. This peptide represented a C-terminal truncation of casein peptide 2, and the kinetics for dephosphorylation of this peptide were similar to those obtained with casein peptide 2 (Table II). The presence of three Glu residues at positions −1 to −3 (peptide 121) decreased the K_m for the reaction by more than an order of magnitude (0.2 vs 2.8 μM) compared with a peptide containing Ala residues (peptide 120) at the three positions (Table V). The V_m values for the two peptides were similar. These results indicate that the Asp residue in angiotensin II and one or more of the three Glu residues in the casein peptide enhance peptide reactivity by decreasing K_m in a fashion similar to that observed with the lysozyme peptide.

Effect of Acidic Residues on Peptide Reactivity with PTP-5. The results of these experiments are shown in Table VI. The presence of the two Asp residues at positions −1 and −5 of the lysozyme peptide (peptide 67-2) decreased the K_m for the reaction by almost an order of magnitude (from 11.4 to 1.8 μM) compared with lysozyme peptide 115 which contains Ala residues at the −1 and −5 positions. The V_ms for the two peptides were similar. In contrast, Asn residues at the two positions (peptide 114) had only a small effect on peptide reactivity. The presence of a single Asp residue at the −1 or −5 position significantly decreased the K_m for the reactions from 11.4 to 3.9–4.0 μM (compare peptide 115 with peptides 110 and 111). The effect of substituting Ala for acid residues in the sequence surrounding the tyr-52 phosphorylation site of α_{s2}-casein was also examined. The three Glu residues at the −1 to −3 position relative to phosphotyrosine decreased the K_m by more than an order of magnitude from 24.7 to 0.6 μM and decreased by the V_m by ~2-fold (compare casein peptides 120 and 121). Taken together, these results indicate that, like rrbPTP-1, acidic residues on the N-terminal side of phosphotyrosine increase peptide reactivity with PTP-5 in an additive fashion by decreasing the K_m for the reaction.

Effect of Other Polar Residues on Lysozyme Peptide Reactivity with rrbPTP-1 and PTP-5. In addition to the two Asp residues, there are four other polar residues at the N-terminal side of the phosphotyrosine residue in the lysozyme peptide sequence. The effects of these residues were examined

by comparing the reactivity of the wild-type lysozyme peptide with that of peptides containing single or multiple Ala's for polar substitutions (Table IV). Single Ala substitutions of the Thr and Ser residues at the −2 and −3 positions (peptides 109 and 68-2, respectively) had no effect on the K_m compared with the wild-type lysozyme peptide (peptide 67-2). Additionally, substitution of the Asp residue at the −1 position with Ala produced an increase in K_m that was not significantly different from the increase in K_m observed when the three residues in the −1 to −3 positions were substituted with Ala (compare peptides 76 and 110 with the wild-type peptide 67-2). This indicates that the Asp residue is the only residue in this region that effects peptide reactivity. Conversely, substitution of the Asp at the −5 position with Ala produced an increase in K_m (1.8-fold) that was significantly smaller than the increase in K_m observed when the three residues at positions −5 to −7 were substituted with Ala (3.5-fold). This suggests that other residues in this region may contribute to peptide reactivity. The best candidate seems to be the Asn at position −7 because of the slight ability of Asn to substitute for Asp residues at the −1 and −5 positions and because substitution of Asn at the −7 position with Ala produced a small increase in K_m (1.6-fold) although this increase was not statistically significant. Substitution of the Thr residue at position −6 had no significant effect on K_m. We conclude that Ser and Thr residues have no effect on peptide reactivity with rrbPTP-1, at least within the context of the sequence surrounding the Tyr-53 phosphorylation site of lysozyme. In contrast, the Asn residue at the −7 position relative to phosphotyrosine may slightly improve peptide reactivity with rrbPTP-1. It is of interest that substitution of this Asn residue with Ala also produced a small increase in the K_m for PTP-5 (compare lysozyme peptides 67-2 and 112 in Table VI.

Comparison with Other Studies of PTP Substrate Specificity. Previous studies have shown that all three PTP classes, transmembrane, cytoplasmic, and dual specificity, can dephosphorylate relatively short (8–20 residue) peptide substrates (Guan & Dixon, 1990; Dunphy & Kumagai, 1991; Cho et al., 1992, 1993; Hashimoto et al., 1992; Ramachandran et al., 1992; Ruzzene et al., 1993). While the present work was in progress, two other studies of the effect of acidic residues on peptide reactivity with PTP's were reported. In one study it was found that a Glu residue at the −1 position and an Asp residue at the −3 position of a peptide based on the Tyr-416 phosphorylation site of *src* increased peptide reactivity with the T-cell PTP ~2-fold compared with Ala at the same position (Ruzzene et al., 1993). The T-cell PTP is another member of the intracellular class of PTP's. These results were based on initial rates measured at a single substrate concentration. Interestingly, an Asn at the −2 position of the peptide also improved peptide reactivity by ~2-fold. In a second study, the presence of an Asp residue rather than an Ala at the −1 position of a peptide based on the Tyr-771 phosphorylation site of PLCγ increased peptide reactivity with the transmembrane PTP, HPTBβ, by decreasing the K_m for the reaction (Cho et al., 1993). In contrast, substitution of an Asp residue for Gly at the −2 position or substitution of Asp for Ala at the +2 position of this peptide had little or no effect on peptide reactivity.

It has also been reported that basic residues decrease peptide reactivity with T-cell PTP and HPTPβ. A Lys residue at the −2 position of a peptide based on the Tyr-393 phosphorylation site of *abl* decreased peptide reactivity with the T-cell PTP 4-fold compared with the corresponding peptide with Ala at the −2 position (Ruzzene et al., 1993). Similarly, Arg or Lys residues at the +2 position of a peptide based on the Tyr-771 phosphorylation site of PLCγ decreased peptide reactivity by increasing the K_m for the reaction 35- and 5-fold, respectively, compared to the corresponding peptide with Ala at this position (Cho et al., 1993). Basics do not inhibit at all positions since an Arg residue at position −1 had no effect on the reactivity of the PLCγ-related peptide with HPTPβ.

Comparison with Protein Tyrosine Kinase Substrate Specificity. Acidic residues on the N-terminal side of tyrosine have also been implicated as positive determinants for substrate recognition by protein tyrosine kinases (Geahlen & Harrison, 1990). Substitution of Glu residues at position −1 or −4 in a peptide based on the Tyr-416 autophosphorylation site of $pp60^{src}$ or Asp residues at the −3 position of the $pp60^{src}$-related peptide or angiotensin II increased the K_m for phosphorylation by protein tyrosine kinases. Kinases influenced by acidic residues include EGF receptor, insulin receptor, $p56^{lck}$, $pp60^{v-src}$, and Y73 P90 (the 90-kDa transforming protein of Y73 virus). The magnitude of the effect of single Asp or Glu substitutions on protein tyrosine kinase K_ms (2–7-fold) is comparable to the magnitude of the effect of single Asp substitutions on the K_ms for PTP-5 and rrbPTP-1 (2–3-fold). It is not known whether substitution of multiple Asp and/or Glu residues produces an additive effect on the reactivity of peptides with protein tyrosine kinases as observed with PTP's.

A significant difference between PTP's and protein tyrosine kinases is that the K_m values (0.3–40 mM) observed with peptide substrates for protein tyrosine kinases are much higher than the K_m values (0.1–2.0 μM) for the best PTP-5, rrbPTP-1, T-cell PTP, and HPTBβ substrates. Additionally, in the one case where it has been tested, the K_m for enolase as a protein tyrosine kinase substrate was 100 times lower than that for the corresponding peptide substrate. This suggests that secondary and tertiary structure may play an important role in substrate recognition by protein tyrosine kinases whereas peptide structure seems to be critical for substrate recognition by rrbPTP-1 and PTP-5.

Summary. The present study is the first to quantitatively relate the reactivity of PTP's toward a protein and its cognate peptide substrate. It was found that short peptides (10–11 residues) possess all or nearly all of the structural features necessary for high reactivity with two intracellular PTP's, rrbPTP-1 and PTP-5. Investigation of structural features necessary for this high reactivity indicated that Asp or Glu residues within four to five residues on the N-terminal side of tyrosine increased peptide reactivity with both PTP's in an additive fashion whereas Asn residues had little or no effect. The effects of the acidic residues, which were as large as 20-fold, may be physiologically significant because many PTP substrates have acidic groups in the vicinity of the tyrosine phosphorylation sites. These results suggest that tyrosine-phosphorylated peptides may be useful reagents for studying PTP substrate specificity.

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