Sequential Dephosphorylation of a Multiply Phosphorylated Insulin Receptor Peptide by Protein Tyrosine Phosphatases†

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ABSTRACT: The question of whether protein tyrosine phosphatases (PTPases) dephosphorylate a multiply phosphorylated peptide in a random or ordered manner was investigated using the synthetic triphosphotyrosyl peptide TRDIY(P)ETDY(P)Y(P)RK, corresponding to the major sites of autophosphorylation of the insulin receptor, as a substrate for four purified PTPases. All four enzymes dephosphorylated the triphospho peptide to produce diphospho, monophospho, and nonphosphorylated forms. Partially dephosphorylated peptides were separated by reverse-phase HPLC, and the di- and monophospho peptides were collected and analyzed by solid-phase sequencing to determine the order of dephosphorylation of the three sites by each of the PTPases. The quantitative analysis of the signals for derivatives of tyrosine and phosphotyrosine generated at positions 5, 9, and 10 of the peptide showed that the low molecular weight human placental PTPase 1B preferentially dephosphorylated the two phosphotyrosines at positions 9 and 10 whereas the integral membrane enzyme CD45 (from human spleen) and the bacterially expressed rat LAR preferentially dephosphorylated the phosphotyrosine at position 5. A second low molecular weight enzyme, termed TCPTPase, did not display any specificity for a particular phosphotyrosyl residue. These results demonstrate that different PTPases exhibit a characteristic pattern of dephosphorylation of the triphospho peptide model substrate, raising the possibility that features in the primary structure surrounding the dephosphorylation site may contribute to substrate specificity.

Protein tyrosine phosphorylation has been recognized in the last 10 years as being intimately associated with signal transduction mediated by various growth factors and oncogenes [for a recent review see Hunter (1989a) and Ullrich and Schlessinger (1990)]. Both receptor and nonreceptor-like tyrosine kinases have been identified. As with serine/threonine phosphorylation, it was originally believed that the regulation of the phosphorylation state of tyrosyl residues in protein is primarily at the level of protein tyrosine kinases. However, recent evidence shows that the phosphorylation state of a protein is finely controlled by the balance between the relative activities of the protein kinases and the family of protein phosphatases (Fischer et al., 1991; Hunter, 1989b; Tonks & Charbonneau, 1989).

Recently a low M_r protein tyrosine phosphatase (PTPase),[1] termed PTPase 1B, was purified to homogeneity (Tonks et al., 1988a,b) and subsequently sequenced (Charbonneau et al., 1988, 1989). Surprisingly, the sequence of this enzyme, although showing no homology to protein serine/threonine phosphatases, was homologous to the intracellular domains of leukocyte common antigen CD45. Subsequently, PTPase activity intrinsic to CD45 was demonstrated (Tonks et al., 1988c, 1990). Further work has shown at least two distinct classes of PTPases. The first class comprises the nontransmembrane enzymes including species similar to that purified from human placenta (PTPase 1B); these are characterized by a single catalytic domain of approximately 35 kDa. Similar enzymes have been cloned from human T cells (Cool et al., 1989), human placenta (Chernoff et al., 1990; Brown-Shimer et al., 1990), and rat brain (Guan et al., 1990). A second class of PTPases typified by CD45 is characterized by two homologous intracellular domains linked to a extracellular receptor motif (Streuli et al., 1988, 1989). Subsequently, cDNAs for additional members of this family of receptor-linked PTPases have been isolated, cloned, and sequenced [Jirik et al., 1990; Kaplan et al., 1990; Krueger, et al., 1990; Matthews et al., 1990; Sap et al., 1990; for review see Alexander (1990)]. It has now been established that PTPases are a complex family of proteins with apparent specificity for the dephosphorylation of phosphotyrosyl residues in proteins. Their properties also suggest that they may be recessive oncogenes (Laforgia et al., 1991).

The identification and characterization of physiologically relevant substrates for protein tyrosine kinases and thus also phosphatases remain a major goal of research in this area. It

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[1] Abbreviations: PTPase, protein tyrosine phosphatase; LAR, leukocyte common antigen related protein; TCPTPase, T cell protein tyrosine phosphatase; BIRK, baculovirus insulin receptor kinase cytoplasmic domain; EDC, *N*-ethyl-*N*'-[3-(dimethylamino)propyl]carbodiimide; MES, 2-(*N*-morpholino)ethanesulfonic acid; PP_i, pyrophosphate; TFA, trifluoroacetic acid; DMS, dimethyl sulfide; TFMSA, trifluoromethanesulfonic acid.

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has been established that at least with artificial model substrates PTPases have extremely high activities that are at least an order of magnitude higher than that of the kinases (Tonks et al., 1988b). This suggests that the activity of PTPases must be tightly regulated for tyrosine phosphorylation events to occur. The existence of multiple types of PTPases in a single cell suggests the possibility that PTPases may recognize specific substrates. However, to date the basis of any specificity for sites of dephosphorylation has not been determined.

In this paper we describe an investigation of the order of dephosphorylation of a multiply phosphorylated peptide by four purified PTPases. As substrate, we have used a peptide corresponding to the major autophosphorylation site of the insulin receptor that was chemically synthesized in the fully phosphorylated form. This peptide contains three tyrosyl residues; in intact insulin receptor, it has been established that conversion of the diphospho to the triphospho form in this segment induces activation of the insulin receptor protein tyrosin kinase (Flores-Riveros et al., 1989; White et al., 1988). The various dephosphorylated forms of this peptide were separated by HPLC. The diphospho and monophospho species generated as a result of phosphatase action were purified and further analyzed by solid-phase protein sequence analysis to quantitate the level of phosphate remaining at the three sites. The results demonstrate that while the four different enzymes used in this study dephosphorylated the triphosphotyrosyl peptide, they can be distinguished by the order in which they dephosphorylate the three sites. While the natural substrate is not yet known for any individual PTPase, it is known that the region of the receptor corresponding to the triphosphotyrosyl peptide used in this study is a substrate for some PTPases and that loss of one specific phosphate from this region of the receptor is sufficient to deactivate it (King et al., 1991). It is therefore of considerable interest to determine the order of dephosphorylation of this triphosphotyrosyl region by individual PTPases, both in the intact insulin receptor and in the peptide.

Experimental Procedures

Materials. The triphospho peptide of the sequence TRDIY(P)ETDY(P)Y(P)RK, corresponding to residues 1142–1153 of the human insulin receptor [numbering system of Ullrich et al. (1985)], was chemically synthesized and purified by Peninsula Laboratories, Inc. (Belmont, CA). The phosphopeptide at 1-nmol scale was synthesized from Boc-amino acids using Merrifield resin and a benzyl protecting group for phosphotyrosine. The peptide was cleaved from the resin using TFMSA/TFA/DMS/*m*-cresol (5:5:3:1 v/v) for 3 h at 0 °C and 1 h at room temperature. Crude peptide was purified using a C_{18} open column, after which the peptide was hydrogenated in 95% aqueous acetic acid and further purified by preparative HPLC. Analaysis of amino acid composition and primary structure confirmed the sequence. The nonphosphorylated peptide corresponding to the above sequence was synthesized by Multiple Peptide Systems, Inc. (San Diego, CA) and by Peninsula Laboratories, Inc. Electrospray ionization mass spectrometry of the nonphospho and triphospho peptides produced multiply charged molecules, from which the calculated molecular weights were 1621.8 and 1861.8, respectively, as predicted. A monophospho peptide was generated by phosphorylation with partially purified BIRK (baculovirus-produced insulin receptor kinase) at a final concentration of 31 μg/mL in a reaction mixture containing 20 mM Hepes (pH 7.4), 10 mM $MnCl_2$, 2 mM dithioerythritol, 100 μM nonphosphorylated peptide, and 250 μM [γ-^{32}P]ATP. The phosphorylation was performed at 25 °C for 2 h and was terminated by addition of trifluoroacetic acid (TFA) to a final concentration of 0.1%. The sample was then loaded directly onto a C_{18} sample cleanup column (Alltech). The column was washed with 0.1% TFA followed by 5% acetonitrile containing 0.1% TFA. The phosphorylated peptide was eluted with 50% acetonitrile/0.1% TFA and dried in vacuum. It was used after it was dissolved in water. The concentration of the phosphorylated peptide was determined from the specific activity of ATP. When an aliquot of this preparation was subjected to the HPLC separation as described below, more than 75% of the radioactivity eluted with the monophosphorylated peptide and about 15% of the radioactivity eluted with the diphospho peptide.

PTPase 1B from human placenta (Tonks et al., 1988a), CD45 from human spleen (Tonks et al., 1990), and the active truncated form of the TCPTPase (Cool et al., 1989; Zander et al., 1991) were purified as described previously. The rat LAR intracellular segment containing the two catalytic domains expressed in *Escherichia coli* was purified as in Pot et al. (1991). The baculovirus containing the cytosolic catalytic domain of the insulin receptor (BIRK) was a generous gift from Dr. O. Rosen and was partially purified up to the Mono Q step from Sf9 cells 72 h postinfection (Villaba et al., 1989).

Protein Tyrosine Phosphatase Assay. The final assay mixture contained 20 mM imidazole (pH 7.0), 6 mM EDTA, 45 mM 2-mercaptoethanol, and the triphospho peptide at the concentrations indicated in the figure legends. The reaction was initiated by addition of phosphatase, and the incubation temperature was 25 °C. At the times indicated in the figure legends, the reaction was terminated by addition of cold TFA to a final concentration of 0.1%. The sample was filtered and injected directly onto the HPLC. For amino acid analysis or peptide sequencing, the peaks corresponding to the triphospho, diphospho, monophospho, and nonphosphorylated peptide were collected directly from the outlet of the detector into Eppendorf tubes and stored at −20 °C. Amino acid analysis was carried out at the Sheldon Biotechnology Centre of the McGill University after acid hydrolysis at 150 °C for 2.5 h using a Pharmacia Alpha Plus amino acid analyzer (Zhu et al., 1987).

All the PTPases were also assayed with ^{32}P-labeled insulin receptor peptide at a final concentration of 20 μM. The reaction was terminated at various times (0–60 min) by addition of an equal volume of 30% acetic acid containing 2 mM PP_i. An aliquot of this was spotted onto P-81 paper, was washed three times with 15% acetic acid containing 1 mM PP_i and once with acetone, and was dried. Radioactivity on the filter paper was quantitated by liquid scintillation counting. Phosphatase activity was calculated from the initial rate of dephosphorylation. One unit of phosphatase activity is defined as the amount of enzyme that causes release of 1 nmol of phosphate in 1 min under the conditions described above.

HPLC Separation of Insulin Receptor Peptide. The various phosphorylated forms of the insulin receptor peptide were separated from the reaction constituents using a 7-μm Aquapore C-18 cartridge (2.1 × 100 mm) with 300-Å pore size. An Applied Biosystems Model 150A HPLC system with a Model 783 absorbance detector was used to monitor the peptide at 276 nm or at 218 nm. The flow rate was 0.4 mL/min. A gradient of acetonitrile in 0.07% TFA (7–25%) was used to develop the column over a 20-min period. All other details are given in the figure legends. All samples were filtered through a 0.22-μm Millex GV4 filter before being injected.

Sequencing and Quantitation of the Phosphotyrosine. The degree of phosphorylation at each tyrosyl residue in the peptide

was quantitatively determined by solid-phase sequence analysis following the method described by Aebersold et al. (1991). The peptides were attached to arylamine-modified membrane disks as described (Aebersold et al., 1990; Coull et al., 1991). A disk of Sequelon-AA was placed on a plastic film or aluminum foil on a heat block at 55 °C. Aliquots of 5 μL of phosphopeptide solution were applied to the disk. Once the solution had dried, another aliquot was applied, and this process was continued until the total sample volume (typically 80 μL of reverse-phase HPLC-purified peptide) had been applied. After the final drying step, the disk was removed from the heat block and wetted with 5 μL of 0.1 M MES, pH 5.0, containing 15% acetonitrile and 10 mg/mL EDC. The coupling reaction was allowed to proceed for 30 min at room temperature. Disks were then washed consecutively with water followed by methanol and either stored at −20 °C or directly applied to the cartridge of a sequenator.

Sequence analysis was performed with a MilliGen/Biosearch Model 6600 sequenator. The standard 36-min degradation cycle was used, and PTH-amino acid derivatives were quantitatively transferred to a Waters MS 600 HPLC system connected on-line containing a SequeTag reverse-phase column 3.9 × 300 mm). PTH derivatives were eluted with a pH 4.8 ammonium acetate buffer/acetonitrile gradient. The separated compounds were identified at 269 nm with a Waters Model 490 multiwavelength detector. To provide for optimal chromatography conditions with respect to peak sharpness and recovery of PTH-phosphotyrosine, the buffer was supplemented with 1 mM phosphate.

Results

HPLC Separation of the Various Phosphorylated Forms of the Peptide. The order of dephosphorylation of sites within the insulin receptor triphosphotyrosyl peptide, TRDIY(P)-ETDY(P)Y(P)RK, by PTPases was examined. An HPLC protocol was developed to separate the various dephosphorylated species, and in addition a novel method for quantitating the degree of dephosphorylation at each site (Aebersold et al., 1991) was utilized.

The time course of dephosphorylation by purified TCPTPase is shown in Figure 1A–D. At zero time there was a major peak of triphospho peptide eluting at approximately 10.4 min. With increasing incubation time this peptide peak decreased, and a new peptide peak with approximately 2 min longer retention time appeared (see peak 2 in Figure 1B). Upon further incubation with the phosphatase, this peptide was converted to peptides with longer retention times (see peaks 3 and 4 in Figure 1C,D). All the peptide peaks were collected, and amino acid analysis confirmed their identical composition (data not shown). Moreover, as phosphotyrosines are not stable to acid hydrolysis, amino acid composition analysis did not yield any information concerning the phosphorylation state of the peptides. The peptide peak that displayed the longest retention time (peak 4) coeluted with the nonphosphorylated peptide (Figure 1E). When the nonphosphorylated peptide was phosphorylated with BIRK, the phosphorylated product eluted at the retention time of peak 3 (see Figure 1F–H). Furthermore, when the peptide was phosphorylated with BIRK and [γ-^{32}P]ATP, radioactivity in the phosphorylated peptide coeluted with peak 3. The stoichiometry of phosphorylation and direct sequence analysis confirmed that the peptide in peak 3 of Figure 1F–H is monophosphorylated, with more than 80% of tyrosine at position 9 being in the phospho form. These data are in agreement with the findings of Stadtmauer and Rosen (1986). From this pattern of elution we anticipated that the peptide in peak 2 may be the diphosphorylated peptide (confirmed below). Thus the HPLC separation illustrated in Figure 1 resolves the tri-, di-, mono-, and nonphospho peptides.

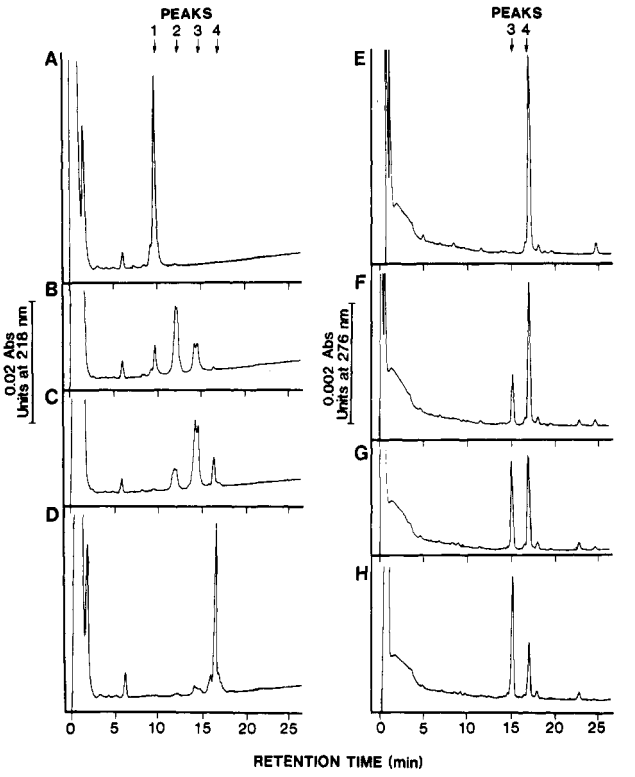

FIGURE 1: Separation of the various phosphorylated forms of the insulin receptor peptide by HPLC. The triphospho peptide (9.5 μM) was incubated with TCPTPase (4 units/mL) at 25 °C as indicated under Experimental Procedures. At times 0 (A), 0.5 (B), 1 (C), and 2 (D) min, 50-μL aliquots of the reaction mixture were removed and added to 200 μL of 0.125% TFA. A total of 200 μL of the filtered sample was injected in the HPLC, and the absorbance at 218 nm was monitored. In (E–H) results are shown of experiments in which the nonphosphorylated peptide (100 μM) was incubated with BIRK as described under Experimental Procedures. At times 0 (E), 20 (F), 35 (G), and 60 (H) min, 100-μL aliquots were removed and added to 500 μL of 0.12% TFA. A total of 50 μL of the filtered sample was injected in the HPLC, and the absorbance at 276 nm was monitored. The peptide in peak 3 of (G) was subjected to sequencing as described in Figure 4, which confirmed that it was a monophospho peptide. The percent compositions of phosphotyrosine at positions 5, 9, and 10 of the peptide in peak 3 of (G) are 1, 87, and 17 respectively. The average retention times for the tri-, di-, mono-, and nonphosphorylated peptides were 10.4, 12.7, 15, and 17.1 min, respectively.

Time Course of Dephosphorylation of the Triphospho Peptide. The time courses of dephosphorylation of the triphospho peptide by the four phosphatases are shown in Figure 2. The triphospho peptide was converted progressively to the diphospho, monophospho, and finally nonphospho form by all the enzymes. This demonstrated clearly that all three phosphates in the peptide were accessible to the phosphatases; however, it did not provide any indication as to the order of dephosphorylation.

Figure 3 displays HPLC profiles of the various phospho peptides from a single time point during the dephosphorylation of the triphospho peptide by the four phosphatases. The profiles of the diphospho and monophospho peptide peaks were nonsymmetrical, suggesting the presence of mixtures of distinct phosphorylated forms. The peaks of triphospho and nonphospho peptides as well as the monophospho peptide formed by the phosphorylation of nonphosphorylated peptide by BIRK (phosphorylated more than 80% at position 9) appeared symmetrical in this separation system (see Figure 1). In addition, each phosphatase generated a characteristic profile of di-

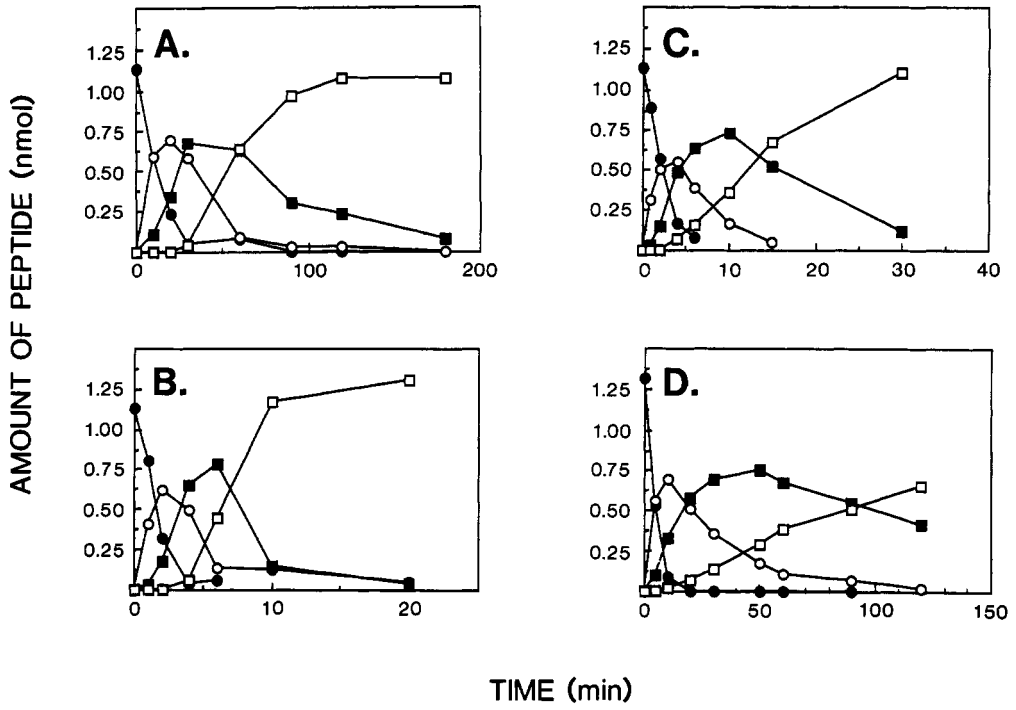

FIGURE 2: Time course of dephosphorylation of the triphospho insulin receptor peptide. The triphospho peptide [28 μM in (A–C) and 16.5 μM in (D)] was incubated with placental PTPase 1B [6 units/mL (A)], TCPTPase [2.8 units/mL (B)], CD45 [4 units/mL (C)], and LAR [0.04 unit/mL (D)] as described under Experimental Procedures. At the times indicated, the reaction was stopped by adding TFA to a final concentration of 0.1%. The peptides in the sample were separated by HPLC, the amounts of triphospho (filled circles), diphospho (open circles), monophospho (filled squares), and nonphospho (open squares) peptides were estimated from the amino acid composition, and the integrated areas under the peaks corresponding to the various peptides were monitored at 276 nm. The estimated specific activities of the PTPases with the triphospho peptide were in the range of 1.6–4.0 μmol mg^{-1} min^{-1}.

phospho and monophospho peptides. This suggested that the heterogeneity of the diphospho and monophospho peptides reflects the preference of the phosphatases for one phosphotyrosyl residue over another.

To test this hypothesis, the diphospho and monophospho peptides were purified by HPLC, and the phosphotyrosine and tyrosine content at each of the three potential dephosphorylation sites was quantitated by solid-phase sequencing. The HPLC profile for PTH-amino acids from a typical sequencing run with the triphospho peptide is shown in Figure 4. Phosphotyrosine was detected in all three positions (5, 9, and 10) as anticipated. In addition, the yields of phosphotyrosine at cycles 5, 9, and 10 (data for cycle 10 not shown) are comparable to that of isoleucine at cycle 4. More importantly, there was virtually no free tyrosine at any of the three positions, clearly demonstrating that there was no artifactual progressive dephosphorylation due to the sequencing chemistry. Therefore, this method is suitable for quantitation of the phosphotyrosine content at each of the three potential sites of dephosphorylation in partially dephosphorylated peptides.

The data in Figure 5 show typical HPLC profiles on the sequencing and quantitation of phosphotyrosine of the diphospho peptide formed by LAR. The figure demonstrates that, under conditions in which all of the phosphotyrosine at position 5 was dephosphorylated, phosphotyrosine at positions 9 and 10 remained relatively unaffected (data for position 10 not shown). Data in Figure 6 summarize the phosphotyrosine content at each of the three positions of the purified diphospho peptides generated by action of the four PTPases under conditions wherein less than 5% of the triphospho peptide was converted to mono- or nonphospho peptide. The results with CD45 (Figure 6C) showed a high degree of dephosphorylation at position 5 compared to positions 9 and 10. This pattern was reminiscent of LAR (Figures 5 and 6D), suggesting that these two receptor-linked enzymes show a preference for the

Table I: Summary of the Dephosphorylation Site Specificity of PTPases

enzyme	order of dephosphorylation of the pTyr residue in the peptide TRDIY(P)ETDY(P)Y(P)RK
CD45 and LAR	5 > 10 > 9
PTPase 1B	9 = 10 > 5
TCPTPase	5 = 9 = 10

phosphotyrosine at position 5 of the peptide. This was further supported by the sequence analysis of the di- and monophosphorylated peptides generated at later time points by these PTPases (data not shown) which showed that dephosphorylation is initiated at position 5 followed by position 10 and finally position 9. The diphospho peptide generated with the placental PTPase 1B (Figure 6A) showed a higher level of phosphotyrosine at position 5 than at positions 9 and 10, with no apparent difference in the degree of dephosphorylation between positions 9 and 10. This indicates that the placental enzyme shows a preference for either of the two phosphotyrosines at positions 9 and 10 over that at position 5. On the other hand, the T cell enzyme (Figure 6B) appeared not to show any preference for any of the three phosphotyrosines. With both placental PTPase 1B and T cell enzyme, the di- and monophospho peptides generated at later time points had the same relative amount of phosphotyrosine at each of the three positions (data not shown), similar to that shown in Figure 6A,B, suggesting that the relative specificity of these enzymes is retained even during the conversion of diphospho peptide to mono- and nonphospho peptides. The specificity for dephosphorylation by the four PTPases is summarized in Table I. It should be noted that, of the enzymes displaying preferential dephosphorylation of a particular phosphotyrosyl residue, in no case was the specificity absolute. Prolonged

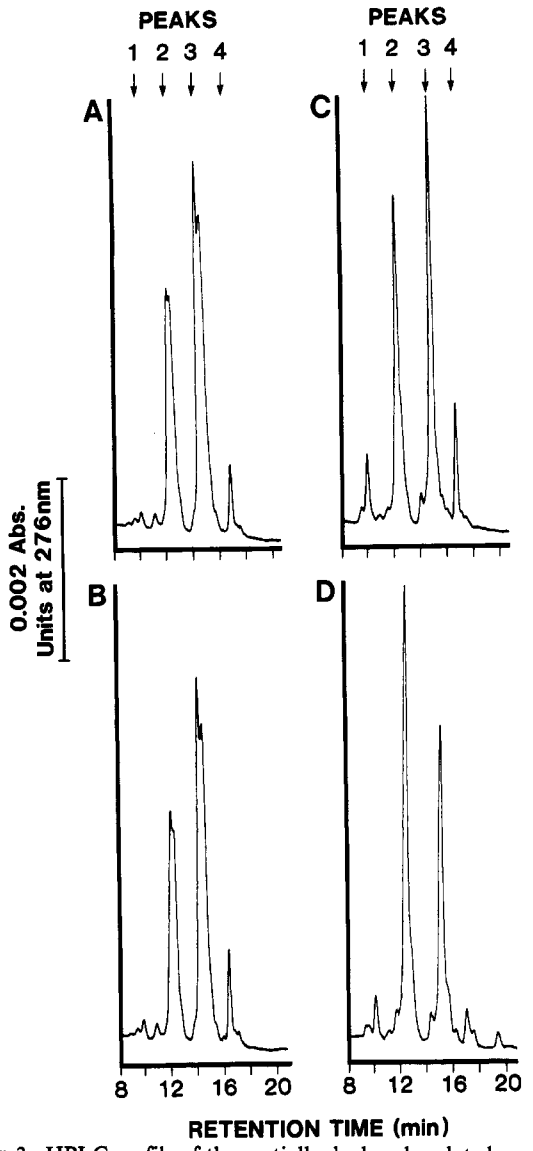

FIGURE 3: HPLC profile of the partially dephosphorylated peptides produced by four PTPases during intermediate reaction times. The reaction conditions were exactly as described in Figure 2. The HPLC traces show the profiles of the mixtures of peptides produced by the action of placental PTPase 1B (A), TCPTPase (B), CD45 (C), and LAR (D) on the triphospho peptide for 4, 30, 4, and 10 min, respectively.

incubation with any of the PTPases studied resulted in quantitative dephosphorylation of all of the phosphotyrosines in the triphospho peptide substrate (Figure 2).

Discussion

The experiments described in this paper were designed to assess whether PTPases display any preferential dephosphorylation of sites within multiply phosphorylated peptides. Because very few natural substrates of protein tyrosine kinases have been fully characterized, few attempts have been made to date to explore this aspect of protein tyrosine dephosphorylation (Shriner & Brautigan, 1984). The multiplicity of the PTPases and the high specific activity of these enzymes studied so far suggest that their activities must be tightly regulated. The kinetic constants for the dephosphorylation of a variety of artificial substrates by the placental PTPase 1B and CD45 suggest that differential substrate recognition may contribute to the control process (Tonks et al., 1988b, 1990).

In instances where multisite phosphorylation of a protein is observed, the sites of phosphorylation are often clustered.

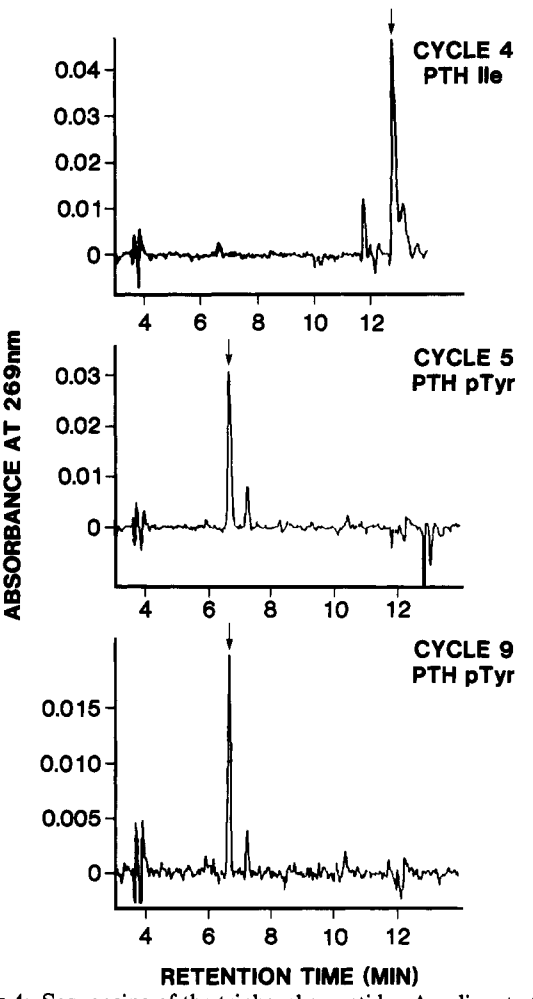

FIGURE 4: Sequencing of the triphospho peptide. An aliquot of the triphospho peptide was subjected to solid-phase sequencing as described under Experimental Procedures. The HPLC profiles at cycles 4, 5, and 9 are shown, and the position of the corresponding PTH amino acid derivative is indicated by the arrow. The profile for cycle 10 was identical to that shown for cycle 9 and is not shown. The size of the peak for PTH-phosphotyrosine decreases with increasing cycles of sequencing due to repetitive stepwise yields of 90–95%.

It appears that both the stoichiometry and sequence of phosphorylation are critical (Roach, 1991). For example, the six or seven sites of insulin-dependent tyrosine autophosphorylation on the insulin receptor are clustered in three segments of the molecule. A six amino acid residue segment referred to as the 1150 domain contains three tyrosine phosphorylation sites (residues 1146, 1150, and 1151). It is clear that conversion of the diphospho to triphospho form in this segment stimulates the protein tyrosine kinase activity of the receptor (Flores-Riveros et al., 1989; White et al., 1988). Recently, the deactivation of the receptor by PTPases was shown to correlate well with the conversion of the triphospho to diphospho form in this segment (King et al., 1991). King and Sale (1990) showed that the 1150 domain is preferentially dephosphorylated compared to other domains of the receptor by rat liver soluble and particulate PTPases. This group also showed that the initial residues to be dephosphorylated by the phosphatases were either one of the two phosphotyrosines at positions 1150 and 1151. However, they were unable to show which of these two phosphotyrosyl residues was dephosphorylated first.

As a model substrate we have used a synthetic peptide, which has three phosphotyrosine residues inserted during the chemical synthesis and which corresponds to the 1150 domain

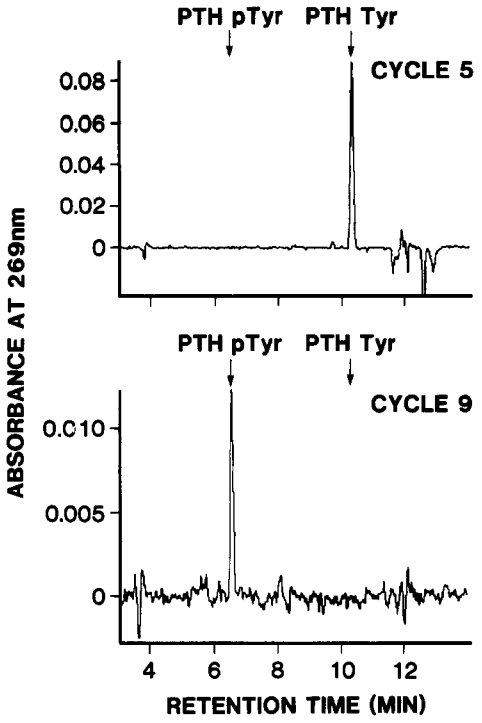

FIGURE 5: Sequencing of the diphospho peptide mixtures generated by the action of LAR. An aliquot of the diphospho peptide fraction purified by HPLC after exposure of the triphospho peptide to LAR under the conditions described in Figure 3 was subjected to sequencing analysis as described under Experimental Procedures. The HPLC profiles at cycles 5 and 9 are shown, and the positions of the PTH derivatives of phosphotyrosine and tyrosines are indicated by the arrows. The profile for cycle 10 was similar to that of cycle 9 and is not shown.

of the insulin receptor. Tyrosines 5, 9, and 10 of this peptide are equivalent to 1146, 1150, and 1151, respectively, in the receptor. We show that this synthetic phosphotyrosyl peptide is a substrate for all the PTPases tested and that all three phosphate groups could be removed by the phosphatases. However, the removal of phosphate was not random. The sequence of dephosphorylation was different for different PTPases (Table I). Methods have been developed to quantitate the amount of phosphotyrosine in each of the three positions of the purified peptide. We found that the human spleen CD45 and the rat LAR preferentially dephosphorylated the phosphotyrosine residues at position 5 followed by position 10, whereas the PTPase 1B from human placenta preferentially dephosphorylated either of the two phosphotyrosines at positions 9 and 10. On the other hand, another low M_r enzyme, TCPTPase, appeared not to show any preference for a particular phosphotyrosyl residue. To our knowledge this is the first time that selectivity of the PTPases for one phosphotyrosine over another has been demonstrated using small multiply phosphorylated peptide. As this work was being completed, Cho et al. (1991) reported the substrate specificity of LAR using a 350 amino acid catalytic fragment of human LAR and a variety of synthetic monophosphorylated peptide substrates including the insulin receptor peptide. They showed that the position 5 monophospho insulin receptor peptide had a higher affinity compared to either position 9 or 10 monophospho peptides. This finding agrees well with our demonstration that phosphotyrosine at position 5 is dephosphorylated first in the triphospho peptide by LAR.

The selectivity of the PTPases for a particular phosphotyrosyl residue appears not to be due to the accessibility of the phosphotyrosyl residues per se, as all the PTPases were assayed

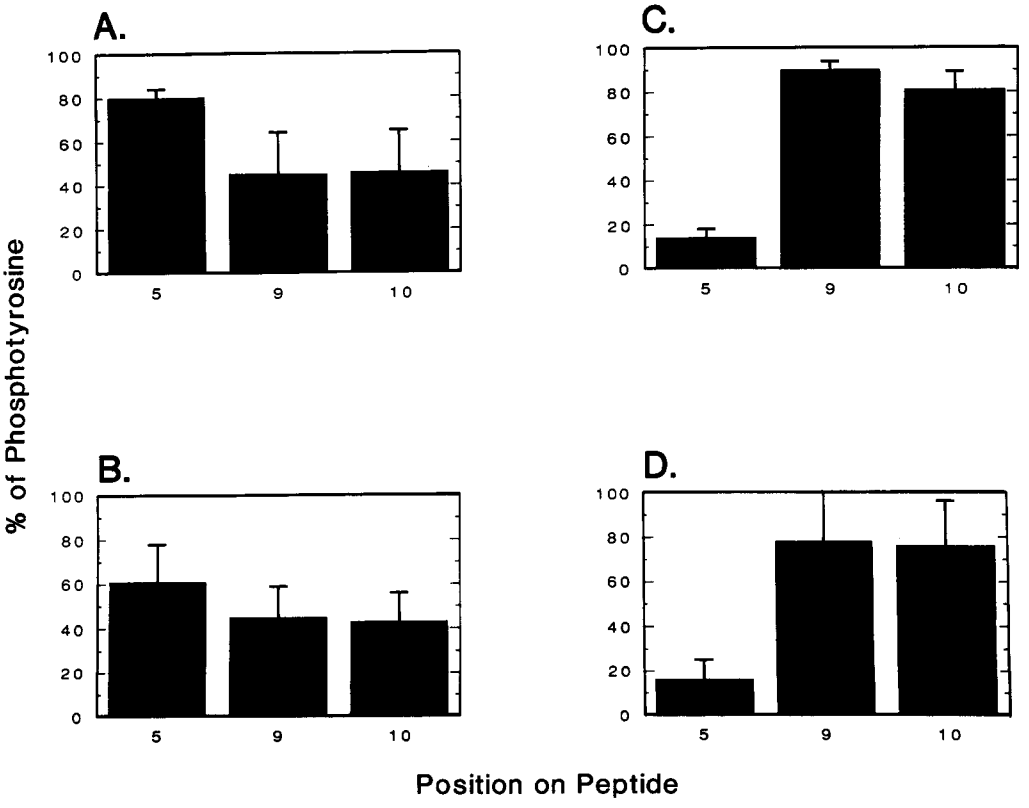

FIGURE 6: Quantitation of the phosphotyrosine content of the diphospho peptides. The diphospho peptide mixture was purified by HPLC from a reaction identical to that in Figure 3 under conditions (first time point after initiating the reaction) wherein less than 5% of the triphospho peptide substrate was converted to mono- or nonphospho peptides. The quantity of phosphotyrosine and tyrosine at each position was calculated from the integrated areas for the PTH derivatives during sequencing as shown in Figures 4 and 5. The mean data and the standard deviation from three independent experiments using PTPase 1B (A), TCPTPase (B), CD45 (C), and LAR (D) are presented.

under the same conditions, and with prolonged incubation times all the phosphotyrosyl residues could be quantitatively dephosphorylated (Figure 2). The substrate peptide has basic residues at the C-terminus and close to the N-terminus and a concentration of acidic residues at the center of the molecule. The role, if any, played by these residues in determining the apparent site specificity of the PTPases remains to be elucidated. It is likely that, as with the protein kinases, the site specificity of the PTPases may also be determined, at least in part, by the nature of the residues surrounding the target phosphotyrosine. Systematic alteration of the amino acids adjacent to the three phosphotyrosines in the above peptide will shed light as to potential recognition elements. In addition, with physiological substrates higher order structures as well as other factors (location, targeting, association with other macromolecules and small molecular metabolites and mediators) may contribute to determining the specificity of the PTPases. Elucidation of the factors determining PTPase specificity will undoubtedly contribute significantly to the understanding of the regulation of various cellular processes modulated by tyrosine phosphorylation.

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