PTPH1 Is a Predominant Protein-tyrosine Phosphatase Capable of Interacting with and Dephosphorylating the T Cell Receptor \( \zeta \) Subunit*

The T and B cell antigen receptors contain multiple copies of an immune tyrosine-based activation motif (ITAMs), which initiate intracellular signals by coupling to several families of protein-tyrosine kinases (PTKs) (1). The activation of this pathway following receptor-ligand interactions results in a transient accumulation of tyrosine-phosphorylated proteins, leading to the induction of cellular effector functions (2, 3). Following TCR engagement, two tyrosine residues within each ITAM are specifically phosphorylated by the Src family of PTKs (2, 4). Once bi-phosphorylated, the ITAMs serve as high affinity binding sites for the ZAP-70/Syk family of PTKs, with each of two Src homology 2 (SH2) domains of Syk/ZAP-70 binding to one of the two phospho-tyrosine sequences (5, 6). The \( \alpha \beta \) T cell receptor (TCR) complex actually comprises up to ten ITAMs that are distributed among the TCR \( \zeta \) and CD3 \( \gamma, \delta, \) and \( \epsilon \) subunits (3). The presence of ten ITAMs is proposed to support signal amplification and/or signal bifurcation (reviewed in Refs. 3 and 4). 6 of 10 ITAMs in the TCR complex are localized in the TCR \( \zeta \) homodimer (three per chain), facilitating the recruitment of multiple ZAP-70 molecules following ITAM phosphorylations (3, 7, 8). There are two predominant tyrosine-phosphorylated forms of \( \zeta \) that migrate with distinct molecular masses of 21 and 23 kDa, and their differential induction are linked to multiple critical biological functions for T cells (3, 8) (reviewed in Refs. 3, 4, and 9).

It is well established that intracellular protein-tyrosine phosphorylations initiated following TCR engagement are transient and this is largely due to the dephosphorylation of key signaling intermediates by protein-tyrosine phosphatases (PTPases) (10–12) (reviewed in Ref. 13 and 14). Although the ITAMs of the TCR \( \zeta \) subunit are transiently tyrosine-phosphorylated, the identity of the PTPase(s) catalyzing their dephosphorylation remains controversial and unclear. To date, incongruous results have been published implicating three PTPases, SHP-1, SHP-2, and CD45, in dephosphorylating the ITAMs of the TCR \( \zeta \) subunit (15–21). SHP-1 is a hematopoietic-restricted cytosolic PTPase defined by the presence of two N-terminal SH2 domains preceding a C-terminal PTPase domain. When compared with normal mice, thymocytes and peripheral T cells from mice deficient in SHP-1 contain numerous signaling proteins which are hyperphosphorylated (22, 23). The phosphorylated ITAMs, the autophosphorylation site of the Src family kinases, and the catalytically activated forms of Syk and ZAP-70 are all considered potential SHP-1 substrates (16, 21, 24, 25). Yet, several reports have shown that SHP-1 does not directly dephosphorylate the ITAMs of the TCR or B cell receptor (BCR) and the substrate specificity of SHP-1 is distinct from the highly conserved ITAM sequence (16, 26–28).

SHP-2 is a ubiquitously expressed PTPase that shares considerable sequence homology with SHP-1, including the pres-
ence of tandem SH2 domains. SHP-2 is generally considered a critical positive regulator of growth factor receptor signaling (29–31). In contrast to its positive regulatory effects, SHP-2 is reported to inhibit TCR signaling by dephosphorylating the 23 kDa-phosphorylated form of TCR (20). Again, other reports have shown that SHP-2 selectively interferes with Erk activation and not with TCR or ZAP-70 phosphorylation (18, 19).

CD45 is a transmembrane PTPase that contains an extracellular domain and two intracellular PTPase domains (reviewed in Ref. 32). CD45 is best known for its ability to directly dephosphorylate the inhibitory tyrosine residue present in the Src family PTKs, resulting in their catalytic activation (32–34). CD45-deficient thymocytes have substantially reduced basal and TCR-inducible levels of TCR and CD3 ε phosphorylation, consistent with the concept that CD45 is a positive regulator of antigen receptor signaling (35). One report has shown that GST-CD45 fusion proteins containing a catalytically inactive derivative of CD45 can bind to the tyrosine-phosphorylated TCR subunit, and wild-type GST-CD45 can dephosphorylate phospho-ξ (15). However, the dephosphorylation of the TCR subunit is still apparent in certain CD45-deficient cell lines (17).

Taken together, the aforementioned reports leave unanswered which PTPases are responsible for dephosphorylating ITAMs. Approaches for identifying such PTPases involve conventional biochemical purification techniques and recent PTPase substrate trap libraries (36). The reaction was terminated after 30 min. The amount of anti-phosphotyrosine mAb was determined with sodium orthovanadate and sodium azide. The plate was washed four times with a Tris-buffered salt solution containing 0.05% Tween-20 (TBST) and then blocked for an hour with 300 μl of 1% bovine serum albumin (carrier). The two active regions were combined from two separate anion-exchange chromatography columns. The beads were eluted off the beads in 10 mM glutathione. The purified phosphorylated ξ molecule was buffer-exchanged into a PTPase assay buffer with PD-10 desalting columns. Removing an aliquot of the kinase mixture at the initiation of the reaction, and calculating the cpm/nmol of phosphate released gives the specific activity according to Equation 1.

\[
\text{cpm of aliquot} \times \text{total vol (500 μl)} = \frac{\text{200 nmol of phosphate}}{\text{activity}}
\]  

(1)

**Protein-tyrosine Phosphatase Assays**—The PTPase assays were performed in a buffer consisting of 50 mM Tris, pH 7.60 containing 0.1% Triton X-100, 2 mM EDTA, 2 mM NaF, and 2 mM 2-mercaptoethanol and protease inhibitors. For quantitative assays, 10 μl of [P32P]labeled substrate was added, and a kinetic assay was performed from 1–10 min. The reaction was terminated by the addition of 50 μl of 100% trichloroacetic acid followed by 50 μl of 1% bovine serum albumin (carrier protein). The amount of 32P remaining in the supernatant was measured in a scintillation counter and the mO of phosphate released per minute were calculated based on the specific activity of the substrate.

Protein amounts were determined with a Lowry-based protein assay.** ELISA-based Protein-tyrosine Phosphatase Assay**—A 96-well plate (Corning) was coated overnight with GST-ξPO4 (6B10.2) at a 1:50 dilution (50 μl/well at 2 μg/ml) in a 0.2 M sodium carbonate, pH 9.10 buffer supplemented with sodium orthovanadate and sodium azide. The plate was washed four times with a Tris-buffered salt solution containing 0.05% Tween-20 (TBST) and then blocked for an hour with 300 μl of 4% bovine serum albumin in TBST. The bovine serum albumin was rinsed from the plate with two TBST washes followed by two washes in a PTPase assay buffer (10 mM Tris-Cl, pH 8.0, 2 mM EDTA, 2 mM 2-mercaptoethanol, 2 mM NaF, protease inhibitors). 100 μl of the assay buffer plus 20 μl of each fraction were added to the individual wells and agitated for 30 min at 37 °C. The plate was subsequently washed four times with TBST. One hundred microliters of anti-phosphotyrosine 4G10, 1 μg/ml concentration of antibody, and 0.4 μl of 1% bovine serum albumin were added for 30 min on ice, washed four times with a Tris-buffered salt solution containing 0.05% Tween-20, and then blotted. The amount of anti-phosphotyrosine mAb bound was determined with standard ELISA assays.

**Chromatographic Separations**—Buffer A: 25 mM Tris-Cl, pH 7.60, 150 mM NaCl, 5 mM/ liter CaCl2, 5 mM/liter MgCl2. Buffer B: 20 mM Tris-Cl, pH 7.60, 2 mM EDTA, 5 mM 2-mercaptoethanol. Buffer C: Buffer B plus 1 mM NaF. Buffer D: 50 mM MES (pH 6.60), 2 mM EDTA, 2 mM 2-mercaptoethanol. Buffer E: Buffer D containing 1 mM NaCl. Buffer F: 50 mM Tris-Cl, pH 7.60, 100 mM NaCl, 5 mM MnCl2, 2 mM 2-mercaptoethanol. Unless otherwise indicated, all buffers were supplemented with aprotinin (1 μg/ml), leupeptin (1 μg/ml), 4 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin, 5 mM benzamidine, and 2 mM sodium fluoride. For initial cellular preparations, between 1 and 5 × 10^9 Jurkat T-cells were washed three times in Buffer A and suspended in phosphate-buffered saline. The cell pellet was resuspended in Buffer B and left on ice for 15 min. The cells were then spun down for 20–30 strokes, left on ice for an additional 20 min and subsequently ultracentrifuged for 2 h (60,000 g × 4 °C). The clarified supernatant was filtered through a 0.22-μm filter containing a glass fiber pre-filter (Spectrum Acrodisc, Gelman Biosciences) and the sample was applied to a 2.5 × 20-cm anion-exchange column linked to an FPLC system (Q-Sepharose, Amersham Biosciences). Proteins retained on the column were eluted with a linear NaCl gradient (Buffer C) at a flow rate of 2.5 ml/min, 10-mI fractions were collected. Each fraction was tested for activity against the TCR-ξ chain using the 32P-labeled GST-ξ protein or the GST construct. Virus was infected into 293 cells and a dose response curve to determine the linear range of phosphate release. For assaying the individual fractions, 10 μl aliquots were incubated with the GST-ξE32PO4 for 1 min, and the specific activity was calculated. The two active regions were combined from two separate anion-ex-
Fig. 1. Jurkat T cell extracts contain PTPases capable of dephosphorylating the phosphorylated TCR ζ subunit. a, an aliquot of a Jurkat T cell homogenate was incubated with a tyrosine-phosphorylated GST-ζ fusion protein for the indicated times. The reaction was terminated, and the fusion protein was resolved by SDS-PAGE and developed by autoradiography. Sodium molybdate (lane 7) and sodium orthovanadate (lane 8) were added at the initiation of the reaction. b, TCR ζ expressed in insect cells was phosphorylated in vitro kinase assays, purified and aliquots were incubated with Jurkat homogenates for the indicated time points. The reaction was terminated and TCR ζ was immunoprecipitated. The precipitates were resolved on SDS-PAGE and immunoblotted with anti-phosphotyrosine mAbs.

RESULTS

Evaluation of Protein-tyrosine Phosphatases in T Cells Dephosphorylating the TCR ζ Subunit—T cells express a large number of PTPases, including 15 of the currently identified intracellular PTPases (42). We attempted to identify the PTPases in Jurkat T cells capable of dephosphorylating the TCR ζ subunit. A Jurkat T cell homogenate was prepared, and the supernatant was obtained following ultracentrifugation. An aliquot of the Jurkat homogenate completely dephosphorylated this substrate within a 3-min incubation period, with no preferential site of dephosphorylation evident (Fig. 1b, lanes 3–6). Again, the enzyme activity was inhibited by the PTPase inhibitor sodium molybdate and/or sodium orthovanadate (lanes 7–8).

Biochemical Purification of Protein-tyrosine Phosphatases that Dephosphorylate TCR ITAMs—To identify the PTPases responsible for the enzyme activities against phospho-ζ, the supernatant from the Jurkat T cell homogenate was applied to an anion-exchange column (Q-Sepharose™). Proteins retained on the column were eluted with a linear NaCl gradient (Fig. 2a). All of the fractions including the flow-through were tested for PTPase activity using a modified ELISA-based PTPase assay. There were two regions of enzymatic activity against the tyrosine-phosphorylated TCR ζ subunit. The majority of the activity eluted within a linear salt gradient between 0.08 and 0.13 M NaCl (fractions 7–16, volume between 260 and 370 ml), while a second region of activity eluted near 0.3 M NaCl, respectively (fractions 46–48, volume between 660–680 ml). These regions were defined as Region-I (fractions 7–16) and Region-II (fractions 46–48), respectively. Region-I from the...
anion-exchange separation exhibited a specific activity of 1.99 pmol/min/microgram while Region-II retained an equivalent activity of 0.23 pmol/min/microgram (Table III). Fractions from the two distinct regions of activity were concentrated and buffer-exchanged in a MES buffer with Centricon Plus-20 ultrafiltration membranes (30-kDa molecular mass cut-off). Using these spin columns, all the enzyme activity remained in the retentate, indicating that the PTPases in question had molecular masses greater than 30 kDa (data not shown). These two distinct buffer-exchanged retentates (Regions I and II) were individually applied to a cation-exchange column (HiTrap SP) (Fig. 2, b and c). Proteins retained on the column were eluted with a linear NaCl gradient and the activity of every fraction was assayed with the ELISA-based assay (Fig. 2b) or the radioactive assay (Fig. 2c and Table III). When subsequently resolved by cation exchange, the first region (Q-pool I) had two separable areas of phosphatase activity, fractions 9–10 and 12–13 (Fig. 2b). Combined, they expressed a specific activity of 34 pmol/min/microgram. In contrast, the second region (Q-pool II) had an enzyme activity that eluted in fractions 16 and 17 (Fig. 2c). Fractions 16–17 maintained a low specific activity of 0.23 pmol/min/microgram (Table III). This second region of enzymatic activity was detected with the radioactive enzyme assay, and was extremely labile. In fact, there were several separations undertaken in which this second enzymatic activity was not detected. As indicated in Table III, these two purification steps yielded an enrichment of 139-fold and 1-fold, respectively.

We were interested in identifying the PTPases present in the enzymatically active fractions. Since earlier reports have suggested roles for SHP-1 and SHP-2 in dephosphorylating the phospho-ζ ITAMs, we examined whether these PTPases were present in our purified fractions (15–21). In addition, a comprehensive analysis of PTPases regulating TCR-signaling has revealed potential roles for PEP, HePTPase, PTP-MEG1, PTPH1, and PTEN (42, 44–46). Several publications have reported that PEP, PTP-PEST, HePTPase, and PTEN do not dephosphorylate tyrosine-phosphorylated ITAMs (reviewed in Ref. 14). The contribution of PTPH1 had not been examined. All the fractions containing and surrounding the regions of enzymatic activity from the two distinct cation-exchange separations were subjected to Western immunoblot analyses with mAbs directed against SHP-1, SHP-2, PTP1B, and PTPH1 (Fig. 3). SHP-1 and SHP-2 (Region-I) were identified in fractions 12–15 and fraction 9 following the cation-exchange separation, respectively (Fig. 3, a and b). Interestingly, the two peaks of enzyme activity included fractions 10 and 12–13, but not fractions 14 or 15 (Fig. 2b). Notably, there is no SHP-2 present in fraction 10, and an abundance of SHP-1 in fraction 14 (Fig. 3, a and b). These results suggested that enzymes in addition to SHP-1 could be responsible for the dephosphorylation of ζ while SHP-2 was unlikely to be responsible for the enzyme activities described. Western blot analyses also revealed that PTPH1 (Region-II) was resolved specifically in fractions 16–17 following a subsequent cation-exchange separation (Fig. 3c). This corresponded directly to the region of enzyme activity (Fig. 2c). These data strongly support the notion that PTPH1 is a PTPase capable of dephosphorylating TCR ζ. Interestingly, PTPH1 was difficult to detect in Jurkat homogenates, suggesting that it is either a low abundance protein, extremely labile, and/or poorly extracted with the described homogenization procedures. An analysis of all the active fractions with additional mAbs indicated that PTP1B was not present (data not shown). Although the isolation of CD45 normally requires detergents extractions, some CD45 was detected in the homogenate (data not shown). However, no CD45 was detected in any of the active fractions. To examine further the potential involvement of CD45 in dephosphorylating TCR ζ, we analyzed CD45-deficient T cells. Notably, these CD45-deficient cells contained a normal level of PTPase activity against phospho-ζ (0.25 pmol/min/microgram) (Table I). These results suggest that the Jurkat T cell line expressed PTPases including SHP-1 and PTPH1 that can be considered candidate PTPases capable of dephosphorylating TCR ζ.

The enzymatically active fractions were also analyzed by mass spectrometry. Using these procedures, we identified a peptide sequence corresponding to SHP-1 in the area encompassing fractions 9–13 (Table IV). We also identified the transmembrane PTPase, PTP-γ, in one purification but this finding was not reproducible.

PTPH1 Is the Predominant PTPase Capable of Complexing Phospho-ζ—The aforementioned biochemical purification strategy suggested that SHP-1 and PTPH1 could directly dephosphorylate TCR ζ. The experiments also suggested that CD45 was not involved in dephosphorylating TCR ζ. However, transmembrane PTPases such as CD45 are largely excluded from the Jurkat homogenate due to their membrane association. Additionally, low abundance PTPases responsible for some of the activities may have remained undetected following mass spectrometry and/or Western blotting. Given these issues, we resorted to a completely independent, previously described PTPase substrate trapping assay (38). The catalytic domain of all PTPases contain an invariant aspartic acid residue that, when substituted with alanine (Asp → Ala), forms an efficient substrate trap and permits the identification of the physiological ligand for a particular PTPase (47). We used a substrate-trapping approach using 47 distinct PTPases. This number represents almost all the PTPases (56 putative) proposed present in the human genome (Table V) (38). Phospho-ζ was coated into individual wells of a 96-well dot blot apparatus. Each well was then probed with a different GST-PTPase fusion protein. Impressively, PTPH1-(D→A) was the only PTPase out of the 47 analyzed that consistently associated with phospho-ζ (Fig. 4a). SHP-1-(D→A), SHP-2-(D→A), and CD45-(D→A) were all incapable of binding to phospho-ζ (Fig. 4c and data not shown). The catalytic domain of PTPH1 also interacted with a non-phosphorylated TCR ζ preparation (Fig. 4b). These findings suggest that PTPH1 and TCR ζ have a unique, previously uncharacterized affinuity and provide the first clearcut demon-
stration that PTPH1 is the predominant PTPase capable of complexing TCR H9256. To verify further these findings, we used GST-fusion proteins comprising the substrate-trap derivatives of PTPH1, SHP-1, or SHP-2 in pull-down assays. COS-7 cells were transfected with Lck and TCR H9256. Forty-eight hours post-transfection, the cells were lysed in Triton X-100-containing buffers. A direct immunoprecipitation of TCR H9256 followed by Western blotting with anti-phosphotyrosine mAbs revealed TCR H9256 as a heterogeneous smear of phosphorylated intermediates (Fig. 4c, lane 4, and Fig. 5a) (8). Importantly, GST-PTPH1(D3A) was the only fusion protein that directly complexed phospho-H9256 in the pull-down experiment (Fig. 4c, lanes 1–3). We could not detect a complex between non-phosphorylated TCR ζ and the GST-PTPH1(D→A) substrate trap. This result suggests that the GST-PTPH1/phospho-ζ association involves a conventional substrate trapping interaction, while PTPH1 and non-phosphorylated ζ have a weak affinity disrupted in the presence of detergents.

PTPH1 and SHP-1 Diminish the Levels of TCR ζ Phosphorylation in Cells through Distinct Mechanisms—To address whether PTPH1, SHP-1, and SHP-2 were regulating TCR ζ phosphorylation in intact cells, we prepared mammalian expression vectors with full-length cDNAs for wild type PTPH1, SHP-1, and SHP-2. COS7 cells were transfected with TCR H9256 and Lck either alone or in combination with PTPH1, SHP-1, or SHP-2. Co-expressing increasing amounts of PTPH1 resulted in a dramatic reduction in the levels of phospho-H9256 without changing the levels of Lck or H9256 expression (Fig. 5, a–d, lanes 1–4). Co-expression of SHP-1 with TCR ζ and Lck also led to a substantial decrease in TCR ζ phosphorylation (Fig. 5, a and b, lanes 6–8). Since SHP-1 can directly dephosphorylate and inactivate Lck, the diminished phosphorylation of TCR ζ is a consequence of reduced Lck function (14). These results could explain the different patterns of phosphorylated TCR ζ intermediates that remain after PTPH1 or SHP-1 co-expression. Thus, PTPH1 initially appears to target lower molecular
weight intermediates while SHP-1 regulates all the higher molecular weight phospho-ζ molecules (Fig. 5, compare lanes 2–4 and 6–8). To examine this issue, we repeated the transfection experiments with TCRζ, Lck and the various PTPases and subsequently analyzed the activation state of Lck with the use of anti-phospho-Src-specific antibodies recognizing the active forms of the Src-family kinases. By directly blotting for active Src, we determined that both PTPH1 and SHP-1 could reduce the levels of active Lck (Fig. 6, a–c, lanes 3–4 versus lane 1 and lanes 7–8 versus lane 5). In contrast to the results with PTPH1 and SHP-1, increasing amounts of SHP-2 had minimal effects on ζ or Lck phosphorylation, when normalized with the expression of TCRζ (Figs. 5 and 6, lanes 10–12 versus 9). Taken together, the aforementioned data strongly suggest that PTPH1 and SHP-1 can directly and indirectly regulate TCRζ phosphorylation.

**Recombinant PTPH1 Directly Dephosphorylates TCRζ**—PTPH1 was purified directly from insect cells using ion-exchange procedures as developed by Tonks and coworkers (Fig. 7a) (48). Interestingly, the elution profile for recombinant PTPH1 on both the anion- and cation-exchange separations was identical to that for the Jurkat homogenate (data not shown). Recombinant PTPH1, ∼80% pure, was incubated with phospho-ζ for 20 s, or 1, 3, 10, and 30 min in the absence or

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**TABLE III**

Purification of phospho-ζ dephosphorylating PTPases from Jurkat T cells

| Purification step | Specific activity pmol/min µg | Protein concentration mg/ml | Total activity pmol/min | Fold enrichment |
|------------------|-------------------------------|-----------------------------|------------------------|----------------|
| T cell homogenate| 0.24                          | 1.0                         | 40,800                 | 1              |
| Anion-exchange    | 1.99                          | 1.16                        | 22,208                 | 8              |
| Cation-exchange   | 33.5                          | 0.089                       | 11,926                 | 139            |
| (Region I)        |                               |                             |                        |                |
| Anion-exchange    | 0.23                          | 0.335                       | 771                    | 1              |
| (Region II)       |                               |                             |                        |                |
| Cation-exchange   | 0.23                          | ND                          | ND                     | 1              |
| (Region II)       |                               |                             |                        |                |

* This preparation exhibited a rapid loss in enzyme activity following two freeze/thaw cycles.

**TABLE IV**

Identification of human PTPases in active fractions

| PTPase | Means of identification | Peptide sequence |
|--------|-------------------------|------------------|
| SHP-1  | Western blot/mass spectrometry | NQLLGPDENAK (309–391) |
| SHP-2  | Western blot             |                  |
| PTP-γ  | Mass spectrometry        | MIWEQNTGIVMITNLVEK (938–956) |
| PTPH1  | Western blot/substrate Trap Library |                  |
presence of PTPase inhibitors (Fig. 7b). Recombinant PTPH1 dephosphorylated TCR ζ within 3 min, with the enzymatic activity substantially blocked by adding the PTPase-specific inhibitors sodium orthovanadate or sodium molybdate (Fig 7, lane 4 versus 7–8). These results demonstrate directly that phospho-ζ is a PTPH1 substrate.

DISCUSSION

The identification of PTPases that specifically dephosphorylate the ITAMs has proven elusive and conflicting results have emerged concerning the contribution of SHP-1, SHP-2, and CD45 in dephosphorylating ITAMs (reviewed in Ref. 9). In this article, we identify PTPH1 as a principal PTPase directly dephosphorylating TCR ζ. Using a biochemical separation scheme with ion-exchange chromatography, we identified SHP-1 and PTPH1 in fractions that exhibited a high specific activity against the tyrosine-phosphorylated TCR ζ subunit. Although these experiments strongly supported the notion that the three PTPases could regulate TCR ζ phosphorylation, the homogenization procedure for isolating these PTPases typically excludes transmembrane PTPases such as CD45 that may also dephosphorylate TCR ζ. Additionally, some of the enzyme activity described could result from PTPases distinct from SHP-1 or PTPH1 that were present in the active fractions. Since

TABLE V

| PTPases used in the substrate-trap assays | Accession number |
|------------------------------------------|------------------|
| BDP-1 | X79568 |
| CD45/LCA | Y00062 |
| CD-1 | L25876 |
| DEP-1 | D37781 |
| EPMA2 | A217063 |
| PAP-1 | D21299 |
| GLEPP-1 | U20489 |
| HPC-PTP | D64053 |
| vH-5 | U27193 |
| I32039 | I32039 |
| IA-2β | AB002385 |
| LAR | Y00815 |
| LC-PTP | D11297 |
| Ly-1 | AF008146 |
| Meg-2 | M83738 |
| MKP-1 | X82777 |
| MKP-2 | HSU21108 |
| MKP-5 | AF176212 |
| mmESP | MMU36488 |
| PE2 | X82676 |
| PTPN/MMae-1 | U92438 |
| PTP-1B | g190741 |
| PTP-α | M34686 |
| PTP-β | X54313 |
| PTP-d1 | XT93510 |
| PTP-δ | L38929 |
| PTP-ζ | L09247 |
| PTP-H1 | M64572 |
| PTP-IA2 | L18983 |
| PTP-κ | L73786 |
| PTP-μ | X55288 |
| PTP-omicron | U71075 |
| PTP-PEST | M93425 |
| PTP-ρ | AF043644 |
| PTP-σ | U88967 |
| PTP-SPR1 | U35284 |
| PTP-xct | M93426 |
| Pyst-1 (MKP-3) | X93920 |
| Pyst-2 (MKP-X) | X93921 |
| SAP-1 | D15304 |
| SHP-1 | X89205 |
| SHP-2 | D13540 |
| STEP | U27831 |
| TC-PTP | M40393 |
| Hz16403 | U14603 |
| VH | L05147 |

PTPases present in the active fractions may have promiscuous activity in in vitro assays, and may not represent the physiologically relevant enzyme, we used subsequently a novel substrate-trapping library comprising the majority of the (47/56) putative PTPases in the human genome (Table V). Not all 56 PTPases are confirmed genes, implying that our panel may actually represent all PTPases. The substrate trapping experiments resulted in the identification of PTPH1 as the predominant and sole PTPase containing a catalytic domain with high

FIG. 4. A substrate-trapping derivative of PTPH1 specifically binds to TCR ζ. a, phosphorylated TCR ζ was coated onto nitrocellulose membranes and incubated with the substrate-trapping derivatives of 47 distinct PTPases. Only a representative sampling including PTP-ESP, PTP-γ, PTP-κ, PTPH1, PTP-IA2, U14603, PTP-ζ, MKP-5, PTP-1B, DEP-1 are shown. GST was used as a negative control. b, both phosphorylated and non-phosphorylated TCR ζ were probed with the substrate-trapping mutant of PTPH1. c, COS7 cells were transfected with the substrate-trapping mutant of PTPH1. c, COS7 cells were transfected with the substrate-trapping mutant of PTPH1. d, the substrate traps comprising the catalytic domains of PTPH1, SHP-1, SHP-2 were used in GST pull-down assays (lanes 1–3). The pull-downs and TCR ζ precipitates were washed, boiled in SDS-sample buffer, and subsequently Western immunoblotted with anti-phosphotyrosine mAbs (lanes 1–4).
substrate specificity for phospho-ζ. Significantly, substrate traps of SHP-1, SHP-2, and CD45 were unable to complex phospho-ζ. Thus, two independent approaches for identifying PTPases targeting phospho-ζ resulted in the discovery of PTPH1. The ability of PTPH1 to dephosphorylate TCR ζ was confirmed in transfection assays and direct enzymatic assays using recombinant PTPH1.

PTPH1 is a 116 kDa cytoskeletal PTPase originally reported to regulate cell cycle progression by dephosphorylating valosin-containing protein (49). It contains FERM and PDZ domains.
followed by a C-terminal phosphatase domain. Transient transfection assays have also suggested a role for PTPH1 in attenuating T cell receptor signaling (50). We show here that PTPH1 can dephosphorylate the TCR ζ subunit in intact cells. This interpretation is strongly supported by the observation that the substrate-trapping derivative of PTPH1 complexes both phosphorylated and non-phosphorylated TCR ζ in dot blot assays. Interestingly, the GST-pull down experiments with the substrate trap of PTPH1 only complexed phospho-ζ, when TCR ζ was prepared from detergent lysates. This suggests that GST-PTPH1 has an extremely low affinity for non-phosphorylated ζ that is disrupted in the presence of non-ionic detergents. We are currently examining the functions of PTPH1 in lymphocytes prior to and following TCR cross-linking. It remains unclear why we were unable to increase consistently the specific activity of the PTPH1-containing fractions during the purification steps. In several instances, we failed to identify PTPH1 in the Jurkat homogenate. We considered several reasons for this problem. First, PTPH1 contains FERM and PDZ domains, and the FERM domain might result in the partitioning of PTPH1 to the cytoskeleton (51). This could result in a substantial proportion of PTPH1 localizing to the pellet following Dounce homogenization and ultracentrifugation. Second, PTPH1 is difficult to detect in Jurkat T cells, suggesting it is a low abundance protein in these cells. Third, PTPH1 is extremely labile and might be sensitive to proteases not blocked by our mixture of inhibitors. We noted that a single freeze/thaw cycle resulted in degradation of PTPH1, as determined by Western blotting. Even recombinant PTPH1, purified in a similar manner from insect cells, degrades easily.

Our substrate trapping experiments indicate that SHP-1 does not complex phospho-ζ. This agrees with reports that SHP-1 does not dephosphorylate the TCR or BCR ITAMs (16, 26, 27). Yet, several reports have suggested that SHP-1 can dephosphorylate ITAMs. We noted that SHP-1 is present in the enzymatically active fractions, suggesting that SHP-1 may dephosphorylate TCR ζ. Moreover, full-length SHP-1 reduced the phosphorylation levels of TCR ζ in COS cells. How can one reconcile these issues? It was possible that additional unidentified PTPases localized in the SHP-1 fractions contributed to the enzymatic activity detected. Alternatively, the activity of SHP-1 toward phospho-ζ may be affected by its own phosphorylation state. SHP-1 has two C-terminal tyrosine residues (52). Although the specificity of SHP-1 would not be affected by its own phospho-

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