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# Substrate specificities of catalytic fragments of protein tyrosine phosphatases (HPTPβ, LAR, and CD45) toward phosphotyrosylpeptide substrates and thiophosphotyrosylated peptides as inhibitors

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**Abstract**

The transmembrane PTPase HPTPβ differs from its related family members in having a single rather than a tandemly duplicated cytosolic catalytic domain. We have expressed the 354-amino acid, 41-kDa human PTPβ catalytic fragment in *Escherichia coli*, purified it, and assessed catalytic specificity with a series of pY peptides. HPTPβ shows distinctions from the related LAR PTPase and T cell CD45 PTPase domains: it recognizes phosphotyrosyl peptides of 9–11 residues from lck, src, and PLCγ with $K_m$ values of 2, 4, and 1 μM, some 40–200-fold lower than the other two PTPases. With $k_{cat}$ values of 30–205 $s^{-1}$, the catalytic efficiency, $k_{cat}/K_m$, of the HPTPβ 41-kDa catalytic domain is very high, up to $5.7 \times 10^7$ $M^{-1}$ $s^{-1}$. The peptides corresponding to PLCγ (766–776) and EGFR (1,167–1,177) phosphorylation sites were used for structural variation to assess pY sequence context recognition by HPTPβ catalytic domain. While exchange of the alanine residue at the +2 position of the PLCγ ($K_m$ of 1 μM) peptide to lysine or aspartic acid showed little or no effect on substrate affinity, replacement by arginine increased the $K_m$ 35-fold. Similarly, the high $K_m$ value of the EGFR pY peptide ($K_m$ of 104 μM) derives largely from the arginine residue at the +2 position of the peptide, since arginine to alanine single mutation at the −2 position of the EGFR peptide decreased the $K_m$ value 34-fold to 3 μM. Three thiophosphotyrosyl peptides have been prepared and act as substrates and competitive inhibitors of these PTPase catalytic domains.

**Keywords:** CD45; HPTPβ; LAR; phosphotyrosyl peptide; PTPase; substrate specificity; thiophosphotyrosyl peptide

The PTPase HPTPβ is a member of the receptor-linked PTPase family, containing an extracellular domain, a transmembrane domain, and a cytosolic catalytic domain (Streuli et al., 1989). It differs from the other seven human PTPases of this class identified to date in that HPTPβ has a single ca. 300-amino acid-long catalytic domain in contrast to two tandem repeats in CD45/LCA (Ralph et al., 1987; Tonks et al., 1988a), LAR (Streuli et al., 1988), and PTPα, γ, δ, ε, and ζ (Krueger et al., 1990). We have recently expressed the tandem repeat catalytic domains of CD45 and human LAR in single (D1) and double (D1D2) forms, purified them, and determined that the phosphoenzyme intermediate in the pure LAR-D1 fragment is catalytically competent in phosphotyrosyl peptide hydrolysis, and its covalent attachment is to the thiolate side chain of the conserved cysteine, Cys 1,522 of LAR (Cho et al., 1992a,b). These results are in agreement with the results obtained earlier by Dixon and colleagues, who detected a phosphoenzyme intermediate and identified a cysteine residue as the nucleophile in the active site using

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*Abbreviations:* PTPase, protein tyrosine phosphatase; pY, phosphotyrosyl; pY(S), thiophosphotyrosyl; IR, insulin receptor; EGFR, epidermal growth factor receptor; PDGFR, platelet-derived growth factor receptor; PSPase, protein serine phosphatase; PLC, phospholipase C; pNPP, *p*-nitrophenyl phosphate.

rat LAR (Guan & Dixon, 1991; Pot et al., 1991; Pot & Dixon, 1992). We have also utilized synthetic phosphotyrosyl peptides of 9–12 residues corresponding to known pY sites in proteins in signal transduction cascades to characterize these PTPases for specificity and selectivity (Cho et al., 1991, 1992b). While LAR-D1 and the D1D2 fragment showed a wide range of discrimination, the relative rank ordering of pY peptide recognition, by the $k_{cat}/K_m$ catalytic efficiency criterion, was very similar between the pure LAR-D1 and the pure CD45-D1D2 catalytic fragments.

The catalytic domain of HPTP$\beta$ was recently expressed in *Escherichia coli* and used for the study of the modulatory effects of various low molecular weight compounds (Itoh et al., 1992) and for the structure–functional study of the enzyme by extensive mutation analysis (Wang & Pallen, 1992). In this study we report expression of the single catalytic domain of HPTP$\beta$, its purification from an *E. coli* overproduction construct, and studies on pY peptides that reveal the PTPase $\beta$ catalytic fragment shows substantially higher affinity for several pY peptides. Also studied was the effect of sequence variations of PLC$\gamma$ and EGFR pY peptides on the affinity of the peptide substrates for HPTP$\beta$. We also report more extensive kinetic data with LAR and CD45 PTPases using additional pY peptides as substrates. Additionally, we have studied three thiophosphoryltyrosyl (pY(S)) peptides as inhibitors and substrates of HPTP$\beta$, LAR, and CD45 catalytic fragments.

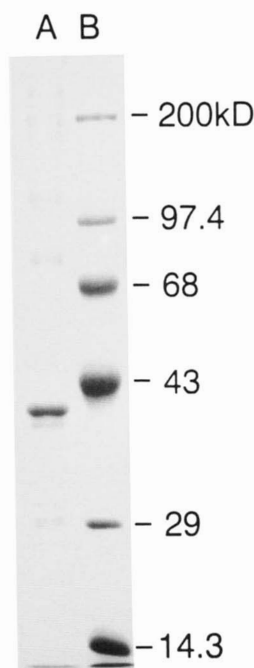


**Fig. 1.** SDS gel of catalytic fragment of purified HPTP$\beta$. Lane A, 41-kDa catalytic fragment of HPTP$\beta$ after the Superdex 75 column step on a 10% polyacrylamide gel in SDS stained with Coomassie blue; lane B, molecular weight standards.

## Results and discussion

### *PTPase$\beta$ catalytic domain*

The 354-amino acid fragment of HPTP$\beta$ was subcloned into an *E. coli* expression vector as described previously (Itoh et al., 1992) and the 41-kDa fragment purified to >80% homogeneity (Fig. 1) by differential ammonium sulfate precipitation, HiLoad Q, MonoS, and Superdex-75. About 1 mg of PTPase catalytic domain was obtained per liter of *E. coli* culture. The expression construct thus resulted in the PTP$\beta$ fragment as 1% of soluble cell protein, requiring ca. 100-fold purification to near homogeneity, as noted in Table 1. The catalytic fragment could also be purified by a parallel protocol, as recently reported (Itoh et al., 1992).

The $\beta$ catalytic fragment was active both in *p*-nitrophenyl phosphate hydrolysis and with the range of phosphotyrosyl peptides noted in Table 2. Several points emerge from the kinetic data. First, the PTP$\beta$ catalytic domain, like its homologs CD45-D1D2 and LAR-D1, has broad specificity for phosphotyrosyl peptides of 9–12 residues from known pY-containing proteins involved in signal transduction cascades, but shows a 100-fold difference in $K_m$ for the pY substrates presented, e.g., the PLC$\gamma$ pY 11-mer $K_m$ of 1 $\mu$M vs. the EGFR pY 11-mer $K_m$ of 104 $\mu$M. Selectivities across the three PTPase catalytic domains for pY peptides also begin to show up. For example, the PTP$\beta$ catalytic domain shows a $K_m$ for the 11-residue peptide containing a pY residue from phospholipase C$\gamma$ that is 230-fold lower than for CD45 and 83-fold lower than for LAR-D1, whereas $V_{max}$ only varies 3-fold across the three enzymes. Analogously, with a nonapeptide encompassing pY$_{527}$ of src, the PTP$\beta$ $K_m$ of 4 $\mu$M is 40-fold lower than for CD45 and 87-fold lower than for LAR-D1, again with only minor variation in $k_{cat}$. Third, the $K_m$ values in the 1–5-$\mu$M range seen with the HPTP$\beta$ catalytic domain are among the lowest values yet reported for any peptide substrate with a PTPase. Coupling the low $K_m$ with the still robust $k_{cat}$ (ca. 56 $s^{-1}$) for the $\beta$ catalytic domain yields $k_{cat}/K_m$ catalytic efficiency values up to $5.7 \times 10^7$ $M^{-1}$ $s^{-1}$, where the diffusional upper limit may be in the $10^8$–$10^9$ $M^{-1}$ $s^{-1}$ range. Wang and Pallen (1992) obtained similar results with the catalytic fragments of HPTP$\beta$ of various lengths. Both RR-src peptide (RRLIEDAEYAARG) and MBP showed $K_m$ and $V_{max}$ values in a range of 2–6 $\mu$M and 10–60 $\mu$mol/min/mg. Our observations, combined with the report by Wang and Pallen (1992), suggest that it is unlikely HPTP$\beta$ will have much lower $K_m$ values for full-length pY proteins if the same $k_{cat}$ values obtain, consistent with recognition of a short sequence of residues around the pY site.

### *Substrate sequence recognition by PTPase$\beta$ catalytic domain*

Given the ca. 100-fold differential affinity of the PTP$\beta$ catalytic domain for the pY 11-mer peptides from the

**Table 1.** *Purification of the cytosolic fragment of HPTPβ*

| Sample | Volume (mL) | Protein concentration (mg/mL) | Total protein (mg) | Total activity (μmol/min) | Specific activity[a] (μmol/min/mg) | Total purification | Yield (%) |
|---|---|---|---|---|---|---|---|
| Cell lysate | 63 | 13 | 819 | 892 | 1.1 | – | 100 |
| 25–45% $(NH_4)_2SO_4$ fraction | 25 | 10.6 | 318 | 722 | 2.27 | 2.1 | 81 |
| HiLoad Q | 110 | 0.644 | 70.8 | 662 | 9.34 | 8.6 | 74 |
| Mono S | 20 | 0.384 | 7.68 | 370 | 48.16 | 44.2 | 41 |
| Superdex-75[b] | 2.5 | 0.306 | 0.765 | 73 | 95.09 | 87.3 | 8 |

[a] Specific activity was measured using *p*-nitrophenylphosphate as substrate.
[b] Only half of the Mono S fraction was loaded onto Superdex-75.

phosphorylation sites of EGFR (104 μM) vs. PLCγ (1 μM), this PTPase and these two peptides seemed to be a reasonable starting point to begin to define pY sequence context selectivity. As a first approximation, two hybrid peptides to interchange the residues on either side of the pY group were synthesized and tested as shown as PLCγ 1 and PLCγ 2 in lines 3 and 4 of Table 3. Replacing the GALYE of PLCγ (773–776) by the LRVA residues (1,174–

**Table 2.** *Kinetic data for LAR-D1, LAR-D1D2, CD45, and HPTPβ using phosphotyrosyl peptides as substrates*[a]

| | Sequence[b] | LAR-D1[c] $V_{max}$ | LAR-D1[c] $K_m$ | LAR-D1[c] $k_{cat}/K_m$ | CD45[c] $V_{max}$ | CD45[c] $K_m$ | CD45[c] $k_{cat}/K_m$ | HPTPβ $V_{max}$ | HPTPβ $K_m$ | HPTPβ $k_{cat}/K_m$ |
|---|---|---|---|---|---|---|---|---|---|---|
| IR-5 | TRDIpYETDYYRK | 96 | 27 | 240 | 72 | 34 | 318 | 95 | 5 | 1,290 |
| IR-9 | TRDIYETDpYYRK | 33 | 280 | 7.8 | | | | 141 | 48 | 200 |
| IR-10 | TRDIYETDYpYRK | 45 | 710 | 4.2 | 134 | 170 | 124 | 116 | 35 | 225 |
| IR-5,9 | TRDIpYETDpYYRK | 52 | 35 | 100 | | | | 294 | 4 | 5,000 |
| IR-9,10 | TRDIYETDpYpYRK | 61 | 302 | 13.5 | | | | | | |
| IR-5,10 | TRDIpYETDYpYRK | 45 | 28 | 108 | | | | 181 | 4 | 3,080 |
| IR-5,9,10 | TRDIpYETDpYpYRK | 72 | 45 | 107 | | | | 265 | 5 | 3,600 |
| IR-5(9,10F) | TRDIpYETDFFRK | 36 | 24 | 101 | | | | 102 | 5 | 1,390 |
| EGFR | TAENAEpYLRVA | 68 | 480 | 8.5 | 136 | 160 | 128 | 43 | 104 | 28 |
| lck394 | EDNEpYTARE | 75 | 170 | 29 | | | | 127 | 2 | 4,320 |
| lck505 | TEGQpYQPQP | 27 | 730 | 2.5 | 66 | 130 | 76 | 174 | 28 | 423 |
| src527 | TEPQpYQPGE | 58 | 350 | 11 | 186 | 160 | 174 | 144 | 4 | 2,450 |
| PDGFRβ-1 | SVDpYVPML | 18 | 236 | 5.1 | 150 | 659 | 34 | 45 | 10 | 306 |
| PDGFRβ-2 | DESVDpYVPMLD | 29 | 287 | 4.2 | 122 | 316 | 58 | 55 | 10 | 374 |
| PLCγ | TAEPDpYGALYE | 57 | 83 | 46 | 168 | 234 | 108 | 84 | 1 | 5,710 |
| cdc2 | IGEGTpYVVYK | 29 | 223 | 8.7 | | | | 81 | 5 | 1,100 |
| ζ-TCR | ATKDTpYDALH | 26 | 93 | 19 | 95 | 208 | 69 | 52 | 10 | 354 |
| FcεR1γ | RNQETpYETLKH | 67 | 103 | 44 | 128 | 107 | 179 | 108 | 18 | 408 |
| pNPP | | 6.2 | 1,700 | 0.24 | 36 | 4,800 | 1.1 | 167 | 1,268 | 9.0 |

[a] $V_{max}$, μmol/min/mg; $K_m$, μM; $k_{cat}/K_m$, $\times 10^4\ s^{-1}\ M^{-1}$. IR, 1,142–1,153 of insulin receptor β chain (White et al., 1985; Tornqvist et al., 1987; Murakami & Rosen, 1991); EGFR, 1,167–1,177 of EGF receptor; lck394, 390–398 of $p56^{lck}$ (Marth et al., 1988); lck505, 501–509 of $p56^{lck}$ (Marth et al., 1988); src527, 523–531 of $p60^{c\text{-}src}$ (Cooper et al., 1986); PDGFRβ-1, 748–755 of PDGF receptor β chain (Kazlauskas & Cooper, 1989); PDGFRβ-2, 746–756 of PDGF receptor β chain; PLCγ, 766–776 of phospholipase Cγ (Kim et al., 1990); cdc2, 10–19 of $p34^{cdc2}$ (Gould & Nurse, 1989); ζ-TCR, 148–157 of T-cell receptor complex ζ chain (E. Reinherz, pers. comm.); FcεR1γ, 71–81 of γ chain of FcεR1, high affinity immunoglobulin E receptor (E. Reinherz, pers. comm.); pNPP; *p*-nitrophenyl phosphate.
[b] The pY residues are aligned. For the multiply phosphorylated IR peptides, pY residues that are preferentially acted on by LAR are shown as bold. As for the IR-5,9, IR-5,10, and IR-5,9,10 peptides, earlier study in this laboratory has shown that the 5 position is dephosphorylated by LAR-D1 before any of the other positions begin to be hydrolyzed. Positions 9 and 10 did not show significant differences in the rate of hydrolysis (Lee et al., 1992). Pot and colleagues also observed preferential dephosphorylation at position 5 of IR-5,9,10 with rat LAR and CD45 (Ramachandran et al., 1992).
[c] Part of the data for LAR-D1 and CD45 (from IR-5 to src527) are reproduced from earlier reports (Cho et al., 1991, 1992b; Lee et al., 1992). These include the data obtained with all the IR peptides, EGFR, lck394, lck505, and src527 phosphotyrosyl peptides.

**Table 3.** *Effect of sequence variations of PLCγ peptide (TAEPDpYGALYE) on kinetic values of HPTPβ PTPase*

| Line | Abbreviation | Sequence | $K_m$ (μM) |
|---|---|---|---|
| 1 | PLCγ | TAEPDpYGALYE | 1 |
| 2 | EGFR | TAENAEpYLRVA | 104 |
| 3 | PLCγ1 | TAEPDpYLRVA | 16 |
| 4 | PLCγ2 | TAENAEpYGALYE | 6 |
| 5 | PLCγ3 | TAEPDpYGRLYE | 35 |
| 6 | PLCγ4 | TAEPDpYGKLYE | 5 |
| 7 | PLCγ5 | TAEPDpYGDLYE | 1 |
| 8 | PLCγ6 | TAENAEpYLAVA | 3 |
| 9 | PLCγ7 | TAEGDpYGALYE | 3 |
| 10 | PLCγ8 | TAEDDpYGALYE | 4 |
| 11 | PLCγ9 | TAEPRpYGALYE | 7 |
| 12 | PLCγ10 | TAEPApYGALYE | 5 |

1,177) of the EGFR pY peptides yields a PTPβ substrate of 16 μM (line 3). The corresponding hybrid pY peptide with the PLCγ residues 766-770 swapped by residues 1,166-1,171 of EGFR yields a $K_m$ of 6 μM for PTPβ (line 4). These data suggest the sequence downstream of the pY residue is more determinative than those just upstream. Whereas the EGFR pY tail raises the $K_m$ some 16-fold in a PLCγ-pY-EGFR hybrid peptide, the PLCγ tail correspondingly in an EGFR-pY-PLCγ peptide lowers the $K_m$ 17-fold (from 104 to 6 μM). The difference between these two tail sequences is most evident in the amino acid residue at the +2 position (R in EGFR and A in PLCγ). This was consistent with data in Table 2. IR-9 peptide (TRDIYETDpYYRK), which has arginine at the +2 position, has the second highest $K_m$ value (48 μM). Positively charged residues were not found at the +2 position of low-$K_m$ substrates.

This was addressed directly by changing A773 to an R773 in the PLCγ variant in line 5 or to a K773 and a D773 in lines 6 and 7 of Table 3. The K and D side chains were well tolerated, but the A → R substitution raised the $K_m$ 35-fold, so it is not just positive charge but the specific cationic guanidinium side chain of arginine that is problematic. An additional test of the same point is displayed in line 8 of Table 3, where the EGFR 1,167-1,177 pY peptide has the R in the +2 position changed to A. The $K_m$ of 3 μM is 34-fold lower than the 104 μM value of the wild-type sequence. Clearly, R at +2 is deleterious.

To begin to assess the preference of HPTPβ for residues at −1 and −2, the four peptides of lines 9-12 of Table 3 were tested. Changes of both charge and bulk are reasonably tolerated at the −2 position; either P → G or P → D substitution increased $K_m$ three- or fourfold (from 1 μM to 3 or 4μM). D → A mutation at the −1 position raised the $K_m$ value fivefold, suggesting the importance of the glutamic acid as a negatively charged residue at that position. Substitution of positively charged arginine residue lowered the affinity even further ($K_m$ = 7 μM), consistent with this suggestion. This sevenfold increase of $K_m$ is the biggest affinity change by a single amino acid exchange except for the A → R mutant at the +2 position (35-fold increase in $K_m$). It is considered that negatively charged residue is most preferred at the −1 position, followed by amino acids with a neutral side chain.

At this juncture the various pY peptides of Tables 2 and 3 can be scanned for sequence motif recognition, and one can conclude first that arginine at +2 is especially problematic. From the insulin receptor peptide data, residues +4 and +5 do not seem crucial for recognition; IR-5,9, IR-5,10, and IR-5,9,10 with negatively charged phosphotyrosyl residues at the +4, +5, and both +4 and +5 positions, respectively, showed $K_m$'s in a range of 4-5 μM, essentially the same value as for IR-5, which has tyrosine residues at both the +4 and +5 positions. Also noteworthy is that the PDGFβ-1 (SVDpYVPML) peptide has essentially the same $K_m$ value as PDGFβ-2 (DESVDpYV PMLD). Additional negatively charged amino acid residues at the −5, −4, and +5 positions do not affect the affinity of the peptide substrate. Many of the low-$K_m$ substrates have D or E at the −1 and/or −3 position; this is a prevalent substitution pattern in many phosphotyrosyl sites. It remains to be determined how much of that is tyrosine kinase vs. PTPase specificity. There are several substrates (e.g., lck394) with positively charged side chains (arginine) at +3 that have low-micromolar $K_m$ values for the 41-kDa HPTPβ fragment.

### *Thiophosphoryltyrosyl substrates*

In addition to screening specific pY peptides by related but distinct PTPase domains to search for distinct recognition elements, the availability of pure PTPase domains in quantity permits evaluation of potentially selective inhibitors and/or alternate substrates. Two variations on the phosphotyrosyl ester functionality that assess phosphate group recognition are the phosphonophenylalanines in which the tyrosyl oxygen is replaced by a $CH_2$ group, and thiophosphoryl analogs in which one of the -$PO_3$ oxygens is replaced by sulfur (Fig. 2). Synthesis and inhibition studies with the phosphonomethyl analogs have recently been reported elsewhere (Shoelson et al., 1991; Chatterjee et al., 1992), and thus we focus here on thiophosphoryltyrosyl analogs of three pY peptides, namely of the C-terminal pY peptide of src, TEPQpYQPGE (Cooper et al., 1986); a diphenylalanine analog of the autophosphorylation site of the insulin receptor tyrosine kinase domain (White et al., 1985; Tornqvist et al., 1987; Murakami & Rosen, 1991); and a pY peptide of PLCγ, TAEPDpYGALYE (Kim et al., 1990). Synthesis and characterization of the pY(S) peptides will be reported elsewhere (Kitas et al., 1993; see Materials and methods).

It is known from kinase studies that ATP-γ-S is generally a slower substrate than ATP, reflecting decreased electrophilicity of the phosphorus atom in -$PSO_2$ vs. -$PO_3$

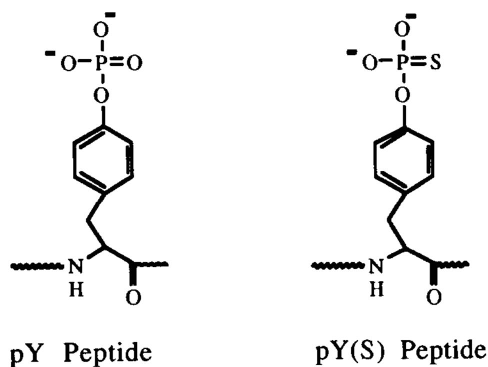


**Fig. 2.** Comparison of phosphotyrosyl (pY) and thiophosphotyrosyl (pY(S)) residues in peptide linkages.

groups for nucleophilic attack. Our initial efforts to use ATP-γ-$^{35}S$ to yield $^{35}SPO_3$ tyrosyl peptides with purified tyrosine kinase $p56^{lck}$ failed because no detectable thiophosphoryl transfer was achieved (Cho & Walsh, unpubl.). cAMP-dependent protein kinase transfers both $-PSO_2$ and $-PO_3$ groups from ATP-γ-S or ATP to histone H1, but in a ratio of ATP-γ-S:ATP 1:10 (Sun et al., 1980). In a conversion of phosphorylase *b* to phosphorylase *a*, phosphorylase kinase utilizes ATP-γ-S at a rate fivefold slower than ATP (Gratecos & Fischer, 1974). Similarly, thiophosphorylated proteins are generally poorer substrates than phosphorylated proteins for phosphatase reactions. Alkaline phosphatase hydrolyzes the ester of phosphorothioic acid 100-fold slower than its oxygen analog (Mushak & Coleman, 1972). Also, in a reaction of phosphorylase *a* with phosphorylase phosphatase, the thiophosphoryl group is hydrolyzed at a rate less than 1% compared to phosphoryl group (Gratecos & Fischer, 1974). Tonks et al. (1988b) reported almost negligible hydrolysis of thiophosphorylated RCM lysozyme by PTPase 1B. This sluggish behavior was also observed with LAR, but to a lesser extent. When the pY(S)-src527 peptide was incubated with LAR-D1, it was enzymatically hydrolyzed at a rate approximately 25% of that observed for the pY-527 peptide. A similarly reduced rate was observed for the pY(S)-IR(9,10F) peptide.

The thiophosphoryl analogs were used as inhibitors of the cognate pY-peptides in colorimetric assays that measure product inorganic phosphate as the malachite green–phosphomolybdate complex. Hydrolysis of the thiophosphoryl peptides, enzymatically or nonenzymatically, to inorganic thiophosphate will not complicate the assay, since the color yield from $PSO_3^{2-}$ was observed to be less than 1.5% of that from an equivalent amount of Pi. Thus, the data of Table 4 show the $K_I$ values of the three thiophosphoryl peptides with the three PTPase catalytic domains. In general it is clear that the pY(S) analogs retain recognition by each of the three PTPases but show higher $K_I$'s than the pY substrate $K_m$'s, within a 3–7-fold $K_m$:$K_I$ ratio for LAR-D1, 1.4–2-fold $K_m$:$K_I$ for CD45, and 3–24-fold $K_m$:$K_I$ for PTPβ. In each instance the pY(S) peptides were competitive inhibitors (data not shown). Compared to the variable loss in affinity with the sulfur substitution on a pY moiety in this study, the phosphonomethylphenylalanine replacements was reported not to lose affinity significantly (Chatterjee et al., 1992).

**Table 4.** *Inhibition constants ($K_I$, μM) for PTPases with pY(S)-IR(9,10F), pY(S)-src527, and pY(S)-PLCγ as inhibitors*[a]

| | LAR-D1 | CD45 | HPTPβ |
|---|---|---|---|
| pY(S)-IR(9,10F) | 168 (24) | 67 (34) | 25 (5) |
| pY(S)-src527 | 1,020 (350) | 270 (160) | 96 (4) |
| pY(S)-PLCγ | 220 (83) | 330 (234) | 3 (1) |

[a] Numbers in parentheses are $K_m$ values (μM) of corresponding pY peptides.

A potent inhibition of HPTPβ was observed with the pY(S) analog of the PLCγ phosphorylation site, TAEP DpYGALYE, the highest affinity substrate ($K_m$ = 1 μM) tested in this study. The pY(S) analog of PLCγ is recognized by PTPase β with a threefold lower affinity than the corresponding pY substrate. The $K_I$ value of 3 μM is the lowest among the reported values with inhibitors toward PTPases. The most potent inhibition of PTPase observed previously was with triphosphonomethylphenylalanine analog of the insulin receptor autophosphorylation site peptide, RDIpYETDpYpYRK, toward PTPase 1B (7 μM of $K_I$) (Chatterjee et al., 1992). More importantly the pY(S)-PLCγ 11-mer peptide inhibits the HPTPβ catalytic fragment 73-fold and 110-fold more potently than the LAR-D1 and CD45 catalytic domains. Whereas okadaic acid (Bialojan & Takai, 1988; Cohen et al., 1989) and microcystin (Honkanen et al., 1990) on the one hand, and FK506 and cyclosporin (presented by their respective immunophilin FKBP and cyclophilin) on the other hand (Liu et al., 1991), are potent and selective inhibitors of PSPases, PP1, PP2A, and PP2B, there are no PTPase-selective or potent inhibitors yet reported. The inhibition of HPTPβ with the pY(S)-PLCγ 11-mer peptide is so far unprecedented in its potency (3 μM of $K_I$) and selectivity (73–110-fold lower $K_I$), and this observation raises the possibility of the use of pY(S)-PLCγ peptide or analogs as PTPase-selective inhibitors for the study of the physiological role of particular PTPases.

## Concluding remarks

Although several transmembrane human PTPases have so far been cloned, to date there is no evidence on the identity of ligands for the extracellular domain and very little information on specific substrates for these putative receptor PTPases. One expects that these PTPases may have broad specificity for any pY-containing protein

brought into apposition. Our previous studies (Cho et al., 1991, 1992b; Lee et al., 1992) on synthetic pY-peptides corresponding to phosphotyrosyl sites in proteins such as tyrosine kinases of the insulin receptor, PDGF receptor, and EGF receptor classes or of the src classes, cell cycle kinases such as CDC2, and from phospholipase C-$\gamma$ revealed a range of $k_{cat}/K_m$ values. The single 41-kDa catalytic domain of PTP$\beta$ shows distinct specificity from LAR and the T-cell specific CD45, and processes several pY-peptides with low-micromolar $K_m$ values. From the pY sequence variation study with the catalytic fragment of HPTP$\beta$, several points begin to emerge. An arginine residue at the +2 position is detrimental, but neutral or negatively charged residues are equally favored at the same position. Amino acid residues with negatively charged side chains are most favored at the $-1$ position followed by those with neutral side chains. Positively charged residue are least favored at the $-1$ position. Amino acid residues at the $-5$, $-4$, $+4$, and $+5$ positions do not play an important role in substrate binding. These data suggest that short peptide sequences in full-length phosphotyrosyl-containing protein substrates may confer in vivo substrate distinctions by the PTPases. A complementary approach to determination of PTPase specificity for defined pY peptide sequences has been undertaken by Dixon and colleagues (Zhang et al., 1993), who used an Ala-scan approach on an EGF receptor phosphopeptide, replacing each residue systematically by Ala to assess catalytic efficiency with recombinant *Yersinia* PTPase and with PTP1.

To assess the physiological role of PTPases specific and selective inhibitors would be useful. Although many potent and selective inhibitors of PSPases are known, there have been no potent PTPase-selective inhibitors yet reported. The data reported here are an initial assessment of the effects of sulfur substitution on a pY moiety. As anticipated electronically, thiophosphoryl groups are now less electrophilic, such that in the three pY(S) peptides, $PSO_2^{2-}$ transfer is slower than transfer of $PO_3^{2-}$. The pY(S) peptides show variable loss in affinity depending on which PTPase is examined.

The $K_I$ value of 3 $\mu$M observed with the pY(S) analog of the PLC$\gamma$ phosphorylation site, TAEPDpYGALYE, toward HPTP$\beta$ is the lowest among the reported values for inhibitors toward PTPases, and it is 73–110-fold lower compared to the $K_I$ value toward the LAR-D1 and CD45 catalytic domains. This potent and selective inhibition of HPTP$\beta$ with the pY(S)-PLC$\gamma$ 11-mer peptide may provide valuable information for the study of the physiological role of PTPases.

## Materials and methods

### *Source and synthetic methods of pY peptides*

The pY peptides in Tables 2 and 3 were prepared by Fmoc solid-phase synthesis as described previously (Kitas et al., 1991; Bannwarth & Kitas, 1992). The preparation of the thiophosphorylated peptides will be described in a separate manuscript (Kitas et al., 1993). Briefly, the synthon for *O*-thiophosphotyrosine, Fmoc-Tyr[PS(OBz)$_2$]-OH, was prepared from Fmoc-Tyr-OH by, first, transient protection as the TBDMS-ester and phosphinylation with $(BzO)_2PN^iPr_2$/tetrazole, followed by oxidation with sulfur. The resulting building block was incorporated in the Fmoc solid-phase synthesis at the thiophosphotyrosine cycle. Deprotection and peptide–resin cleavage were performed with a TFA/thiophenol ($H_2O$) mixture.

### *Assays: Assessment of purity of thiophosphoryl peptides*

Except for purification purposes, which used *p*-nitrophenyl phosphate as a substrate, the enzyme activities were determined by measuring released inorganic phosphate using the malachite green reagent as described (Cho et al., 1991, 1992b). For the HPTP$\beta$ assay, the reaction was initiated by addition of HPTP$\beta$ to a mixture containing pY-substrate, 0.1 M Hepes (pH 7.6), 2 mM EDTA, and 10 mM dithiothreitol (DTT). The reaction was terminated by addition of 0.9 mL of malachite green reagent. The absorbance at 660 nm was then converted to the quantity of Pi. For substrates with $K_m$ values lower than 20 $\mu$M, a modified malachite green reagent was used to increase the sensitivity. Briefly, 0.8 mL of reaction mixture was quenched by addition of 0.2 mL of 4× malachite green reagent, which was prepared by mixing malachite green (101.3 mg) in 4 N HCl (56.3 mL) and ammonium molybdate (6.3 g) in 4 N HCl (75 mL). The reagent was filtered and kept at 4 °C and used for no more than 3 days. The purity of the thiophosphorylated peptides was assessed by analytical high-performance liquid chromatography and capillary zone electrophoresis. The pY(S)-peptides contained less than 10% of unphosphorylated peptides, which were produced during purification. The identity of the thiophosphorylated peptides was assessed by ion-spray mass spectroscopy and protein sequencing. The concentrations of the thiophosphorylated peptides were measured by treatment of the peptides with excess LAR-D1 followed by malachite green assay. The results were confirmed with lower accuracy by analysis of the $^1$H-NMR spectra of the pY(S)-peptides. Inhibition assays were carried out in a buffer containing 100 mM imidazole, 2 mM EDTA, and 10 mM DTT at 25 °C. Rates of hydrolysis of the thiophosphotyrosyl peptides were determined under identical conditions by measuring the released inorganic thiophosphate as described above.

### *Purification of LAR and CD45*

LAR and CD45 were purified as described (Cho et al., 1991, 1992b).

### *Purification of HPTPβ*

The crude lysate obtained from cultures of *E. coli* BL21 (DE3) carrying the pET-cytosolic HPTPβ fragment-containing plasmid (Itoh et al., 1992) was fractionated with ammonium sulfate, and the 25–45% fraction was dialyzed against buffer A. The dialyzed sample was loaded on a HiLoad Q-Sepharose 16/10 column (Pharmacia), washed with buffer A, and eluted with a gradient of 1 M NaCl buffer A. Glycerol was added to the concentrated active fraction (phosphatase activity determined by *p*-nitrophenyl phosphate assay) to a concentration of 33% and was then dialyzed against buffer B. Glycerol addition prevented precipitation of protein during dialysis. The sample was then applied to a Mono S 10/10 column (Pharmacia), washed with buffer B, and eluted with a gradient of 1 M NaCl buffer B. The active fractions were concentrated (Centriprep 30 and Centricon 30, Amicon), made to a glycerol concentration of 33%, and stored at −80 °C. The sample was thawed rapidly, applied to a Superdex-75 column (Pharmacia), and then eluted using buffer C. Active fractions were detected in the elution volume (9–11 mL), concentrated (Centricon, Amicon), made to 33% glycerol, and stored in aliquots at −80 °C.

Buffers were used as follows: buffer A, 33 mM Tris-HCl, 2.5 mM EDTA, 10 mM β-mercaptoethanol, pH 8.5; buffer B, 50 mM HEPES, 2.5 mM EDTA, 10 mM β-mercaptoethanol, pH 8.0; buffer C, 50 mM HEPES, 2.5 mM EDTA, 10 mM β-mercaptoethanol, 0.4 M NaCl, 1% glycerol, pH 8.0.

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