Interconversion of the Kinetic Identities of the Tandem Catalytic Domains of Receptor-like Protein-tyrosine Phosphatase PTPα by Two Point Mutations Is Synergistic and Substrate-dependent*

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Kah Leong Lim, Prasanna R. Kolatkar, Kwok Peng Ng, Chee Hoe Ng, and Catherine J. Pullen§

From the Cell Regulation Laboratory, Institute of Molecular and Cell Biology, 30 Medical Drive, Singapore 117609 and the §Bioinformatics Center, National University of Singapore, 5 Lower Kent Ridge Road, National University Hospital, Singapore 119074, Republic of Singapore

The two tandem homologous catalytic domains of PTPα possess different kinetic properties, with the membrane proximal domain (D1) exhibiting much higher activity than the membrane distal (D2) domain. Sequence alignment of PTPα-D1 and -D2 with the D1 domains of other receptor-like PTPs, and modeling of the PTPα-D1 and -D2 structures, identified two non-conserved amino acids in PTPα-D2 that may account for its low activity. Mutation of each residue (Val-536 or Ile-644) in D2 resulted in a significant increase in its catalytic activity. Mutation of both residues resulted in a 100-fold increase in D2 activity. These results demonstrate the existence of a kinetic interconversion mechanism for the tandem catalytic domains of PTPα. This mechanism is likely to be a general property of receptor-like PTPs, and may offer additional regulatory control over the activity of PTPα.

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§ To whom correspondence should be addressed. Tel.: 65-874-3742; Fax: 65-779-1117; E-mail: mcbcp@imcb.nus.edu.sg.

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higher intrinsic activity of PTPα-D2 compared with other D2 domains and the lower activity of PTPα-D1 compared with other D1 domains (19). Structure-function studies of PTPα-D2 present a unique opportunity to assess the minimal sequence requirements that might distinguish the characteristic catalytic properties of the homologous D1 and D2 domains. Most of the highly conserved and invariant residues among the tyrosine-specific PTPs are found within or near the catalytic cleft and are involved in interaction with phosphorytrosine or in actual hydrolysis (12, 13). Mutation of many of these residues impairs PTP activity (8, 14, 25, 26). The lack of certain of these apparently critical residues in PTPα-D2 suggests that its low activity may simply be due to defective substrate binding or catalysis. If so, this would imply a non-catalytic role for D2 rather than an enzymatic function. To investigate this possibility, we have mutated two atypical residues in PTPα-D2 to conform to the corresponding amino acid found in all other tyrosine-specific PTPs with activity. One such residue is the putative general acid of D2 necessary for formation of the thioephosphate intermediate. The other is a residue that, in PTP1B, is located at the top of the catalytic cleft where it interacts with phosphorytosnine of the substrate (13). The in vitro and in vivo activities of the D2 single and double mutants, as well as those of D1 single and double mutants possessing wild-type D2 residues in these positions, have been analyzed.

**EXPERIMENTAL PROCEDURES**

**Molecular Modeling**—Modeling of structures was performed using LOOK (Molecular Applications Group). Sequences of D1 and D2 were initially aligned to the target sequence of PTP1B (sequence identities of 47 and 41%, respectively), and the structure was subsequently modeled based on the algorithm of Lee and Subbiah (the algorithm uses self-consistent ensemble optimization to determine the global minimum structure resulting in the location of side chains with high accuracy) (27). The target structure was the complexed form of PTP1B (215S) with phosphorytrosine-hexapeptide (13).

**Expression Plasmids**—Numbering of the PTPα amino acid sequence is according to Krueger et al. (28). The bacterial expression plasmids pGEX-KG containing PTPα-D1 or -D2 have been described (19), and they served as template for polymerase chain reaction site-directed mutagenesis. For the WPD mutants, the forward and reverse primers were 5′-ACCAGCTGGCCGAATTTGTTGGTGTG-3′ for D1(382E), 5′-ACACCCGGCACCTTTTGGGTT-3′ for D1(382E), 5′-CATGGCTGGCTGCTGAGGGCACTC-3′ for D2(671D), and 5′-CATGGCTGGCTGCTGAGGGCACTC-3′ for D2(671D). For the KRNY mutants, the reverse primer sequences were 5′-CAGTGAGCCTAGTGGTTACTGTAACAAGCTGATATTATTTTCGACACATGTTCAGGCTACACTGGTTGGTGTG-3′ for D1(Y243F) and 5′-CAGTGAGCCTAGTGGTTACTGTAACAAGCTGATATTATTTTCGACACATGTTCAGGCTACACTGGTTGGTGTG-3′ for D2(V536Y). The pGEX-KG-PTPα-D1(Y243F/V536D) was constructed by removing appropriate restriction fragments from pGEX-KG-PTPα-D1(D382E) and pGEX-KG-PTPα-D1(Y243V) and assembling them together so that they contained the double mutation. A similar strategy was used in the construction of pGEX-KG-PTPα-D2(V536D/Y243F) (30). The plasmids pXJ41-PTPα-D1(C704S) and pXJ41-PTPα-D2(C704S) were constructed by replacing D1 or D2 within pXJ41-PTPα-neo with a corresponding restriction fragment encompassing D1(C414S) or D2(C704S) that was derived from pXJ41-PTPα-D1(C414S/D2(C704S)-neo. The Cys to Ser mutations are denoted in the figure legends as a subscript S following the domain containing the mutation. These plasmids and those containing wild-type PTPα (pXJ41-PTPα-neo) (29) or PTPα-D1(C414S/D2(C704S)-neo) (19) were subsequently used in site-directed mutagenesis and expressed using primers described previously (23). Phosphatase activity was plotted against substrate concentration in the form of a Lineweaver-Burk plot and manually extrapolated to determine K<sub>m</sub> and V<sub>max</sub> values.

**Cell Culture and Transient Transfections**—COS-1 cells were obtained from American Type Culture Collection (Rockville, MD). Cells were transfected with 2–4 μg of plasmid DNA by liposome-mediated transfection with 10 μl (1 mg/ml) (60-mm dishes) or 30 μl (1 mg/ml) (100-mm dishes) of Lipofectin or LipofectAMINE reagent (Life Technologies, Inc.) for 6 h as described by the manufacturer and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and penicillin/streptomycin at 37 °C. To determine confluent monolayers of COS-1 cells were trypsinized and replated in 60- or 100-mm tissue culture dishes and incubated for 16 h until 50–70% confluency. Cells were transfected with 2–4 μg of plasmid DNA by liposome-mediated transfection with 10 μl (1 mg/ml) (60-mm dishes) or 30 μl (1 mg/ml) (100-mm dishes) of Lipofectin or LipofectAMINE reagent (Life Technologies, Inc.) for 6 h as described by the manufacturer and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum for an additional 18 h prior to harvesting. The empty expression plasmid pXJ41neo was used to normalize the amount of DNA in each transfection. Equivalent amount of the various forms of PTPα were expressed.

**Western Blots, Immunoprecipitations, and Kinase Assays**—The preparation of cell extracts and subsequent Western blot procedures have been described (30). Membranes were immunoblotted with anti-PTPα-D1 antisera (no. 2205, raised against a GST-PTPα fusion protein containing the first catalytic domain of PTPα (1:1000) and followed by goat anti-rabbit IgG conjugated to peroxidase (Sigma) (1:2500), anti-p59<sup>phos</sup> monoclonal antibody (Transduction Laboratories) (1:300) followed by goat anti-mouse IgG conjugated to peroxidase (1:2500), or peroxidase-conjugated anti-phosphotyrosine antibody (Transduction Laboratories) (1:2000). Immunoblots were developed using the ECL system (Amersham Pharmacia Biotech). For immunoprecipitation of p59<sup>phos</sup>, anti-p59<sup>phos</sup> (FYN3, Santa Cruz) was added to the cell lysates (1 μg of total protein) and incubated for 2 h at 4 °C. Protein A cell suspension (Sigma) was then added and mixed at 4 °C for 2 h. After low speed centrifugation, the immunoprecipitates were washed twice each with lysis buffer and once in 2× kinase assay buffer containing 10 mM Pipes (pH 7.0), 5 mM MnCl<sub>2</sub>, and 0.5 mM dithiothreitol. Part of the immunoprecipitates were used in kinase assays to measure p59<sup>phos</sup> autophosphorylation as described previously (30). Other portions of the immunoprecipitates were probed for p59<sup>phos</sup> as described above. The p59<sup>phos</sup> level, phosphorytosnine content, and kinase activity were quantitated using a GS700 Bio-Rad densitometer.

**RESULTS**

**Modeling of the Catalytic Domains of PTPα**—Alignment of the amino acid sequences of the catalytic D1 domains of 16 active mammalian receptor-like PTPs shows that they possess 42 invariant residues, highlighted in the amino acid sequence of PTPα-D1 (Fig. 1A). No D2 domain of these RPTPs possesses all 42 invariant residues, although PTPα-D2 is only lacking 3 of them: a tyrosine at position 536, a leucine at position 549, and an aspartate at position 571 (Fig. 1A). In fact, all of the D2 domains examined are lacking the corresponding tyrosine and aspartate residues, suggesting that the substitution of these residues may be a common denominator that, in the absence of other obvious defects (for example, the substitution of the essential cysteine residue in the active site of PTPγ, PTPζ, and PTP-OST), accounts for low D2 activity. Furthermore, the counterpart Tyr in the KNRY motif and the Asp in the WPD motif of non-receptor PTP1B are involved in interactions with the substrate. The crystal structures of PTP1B complexed with phosphopeptide shows that the corresponding invariant tyrosine are maintained in Dubeczko’s modified Eagle’s medium supplemented with 10% fetal calf serum and phosphotyrosine of the substrate (13). This tyrosine is one of several hydrophobic, conserved residues that form the recognition site for phosphotyrosine. In the PTP1B structure, the invariant aspartate (Asp-181) is found in the movable WPD loop and is involved in interaction with phosphotyrosine where it acts as a general acid to facilitate phosphoester hydrolysis. The involve-

**Kinetic Interconversion of PTPα-D1 and -D2**

450-μl reactions containing 50 mM sodium acetate (pH 5.5), 0.5 mg/ml bovine serum albumin, and 0.5 mM dithiothreitol. Dephosphorylation of pNPP was measured in
The substitution of these two invariant residues in PTPα-D2 affected D2 structure, as modeled in Figure 1B. Although the x-ray structure of PTPα-D1 has been reported, it is not complexed with substrate, so models were prepared based on the structure of PTP1B complexed with phosphopeptide. Inspection of all three superimposed structures showed that the molecules were largely similar. One area of interest and notable difference among the structures was the WPD loop region. The carboxylate group of Asp-382 in PTPα-D1 sits close to the phosphate (approximately 4 Å), whereas the carboxylate group of Glu-671 in the WPE loop of PTPα-D2 sits about 7.45 Å from the phosphate, likely due to the larger glutamate side chain having steric hindrance as well as repulsive forces from adjacent negative charges within the active site pocket (Fig. 1B, inset). The distances between these carboxylate groups and the phenolic oxygen are 3.7 and 6.94 Å, respectively (not shown). The aspartate thus fits within the active site, whereas the glutamate is forced to remain at a distance from active site residues.

To see if the substitution of these two invariant residues in PTPα-D1 and -D2 affected D2 structure, we modeled PTPα-D1 and -D2 (Fig. 1B). The modeling algorithm predicts an exact superimposition of the main chain atoms of the D1 and D2 domains, and only the side chain atoms of Asp-382 of PTPα-D1 and Glu-671 of PTPα-D2 differ. Left, the overall catalytic domain structure with the boxed region showing the position of the phosphotyrosine and its relation to the side chains of Asp-382 of PTPα-D1 and Glu-671 of PTPα-D2. Right, magnification of the boxed region showing the distance between the carboxylate groups of Asp-382 (Asp) or Glu-671 (Glu) and the phosphate ion of phosphotyrosine. The proximity of the carboxylate group of Asp and the phosphate group would allow hydrogen bonding and proton donation to the phenolic oxygen of phosphotyrosine, as well as the subsequent activation of the nucleophilic water in the hydrolysis of the phosphoenzyme intermediate.
Kinetic Interconversion of PTPα-D1 and -D2

PTPα-D1 and -D2 Mutants—The roles of Val-536 and Glu-671 of PTPα D1 and D2 toward pNPP and RR-src, respectively, were evaluated by mutagenesis of their different putative general acids to that present in the counterpart domain, in the case of the peptide substrate RR-src, the D1 and D2 activities are still quite distinct even after mutation. This is especially true for D2, where the kinetics of RR-src dephosphorylation by the wild-type D1 (Table 1). The latter contrasts with a report that this same mutation in PTPα-D1(D382E) had a similar effect on the kinetics of RR-src dephosphorylation by the wild-type and D1(D382E) mutants (mutant D1(D382E)). Toward pNPP, the PTPα-D1(D382E) mutant had a 7-fold increase in Km and an 8-fold decrease in Vmax relative to wild-type PTPα-D1, resulting in a turnover number and a catalytic efficiency ratio only 2-3-fold higher than wild-type PTPα-D2. However, toward RR-src, the PTPα-D1(D382E) mutant had an essentially unchanged Km relative to wild-type PTPα-D1, whereas the Vmax increased 200-fold. Despite this, kcat and the catalytic efficiency ratio of this mutant D1 were still 54- and 136-fold higher than those of wild-type D2. The D1 mutant containing alanine rather than an acidic residue in position 382 (D382A) exhibited similar Km values similar to those of the D382E mutant for both substrates but had a further reduced rate of activity (Table 1). The D1(D382A) mutant and wild-type D1 showed no essential difference in Km values for RR-src, although the Km of the mutant for pNPP was about 5-fold higher than that of wild-type D1 (Table 1). The latter contrasts with a report that same mutation in PTPα-D1 has virtually no effect on the Km for pNPP (24). We do not know the reason for this difference, but in our experiments the mutation of Glu-671 to Ala in D2 had a similar effect on the catalytic efficiency of pNPP dephosphorylation (Table 1). The catalytic efficiency ratios of PTPα-D1 D382A were about 560- and 6200-fold lower than wild-type D1 for pNPP and RR-src, respectively, consistent with a role of Asp-382 as a general acid in catalysis.

The above results suggest that in the case of pNPP, PTPα-D1 and -D2 can be induced to behave more, but not entirely, like the other catalytic domain (i.e., D1 like D2 and vice versa) by mutation of their different putative general acids to that present in the counterpart domain, but in the case of the peptide substrate RR-src, the D1 and D2 activities are still quite distinct even after mutation. This is especially true for D2, where the kinetics of RR-src dephosphorylation by the wild-type and E671D forms are virtually the same.

Mutation of Glutamate 671 of PTPα-D2 to Aspartate or Alanine—To test the theory that proton donation to the phenolic oxygen of phosphotyrosine by the more distant hydroxyl moiety of glutamate in wild-type PTPα-D2 would be less catalytically favorable than from a closer hydroxyl moiety of aspartate, Glu-671 was mutated to either Asp or Ala (E671D and E671A, respectively). The kinetic parameters of activity of these PTPα-D2 mutants were assayed toward pNPP and the RR-src phosphotyrosyl peptide. As predicted, the E671D substitution affected PTPα-D2 activity toward pNPP, resulting in an enzyme with a 10-fold increased turnover number (kcat) intermediate to those of wild-type PTPα-D1 and -D2 and with an overall 4-fold decrease in catalytic efficiency ratio (kcat/Km) (Table 1). In contrast, the E671D substitution had surprisingly little effect on the kinetics of RR-src dephosphorylation by PTPα-D2 (Table 1), indicating that Glu-671 in wild-type D2 is not responsible for the very low activity toward this substrate. Consistent with a role for Glu-671 as a general acid in catalysis, the PTPα-D2 E671A mutant exhibited less favorable Km values than wild-type D2, with a 15-fold reduction in the catalytic efficiency ratio of pNPP dephosphorylation and such low activity toward RR-src that it could not be reliably measured (Table 1).

Mutation of Aspartate 382 of PTPα-D1 to Glutamate or Alanine—The mutation of Asp-382 to Glu or Ala in PTPα-D1 (D382E or D382A, respectively) had pronounced effects on the kinetics of dephosphorylation of pNPP and RR-src peptide (Table 1). Toward pNPP, the PTPα-D1(D382E) mutant exhibited a 7-fold increase in Km and an 8-fold decrease in Vmax relative to wild-type PTPα-D1, resulting in a turnover number and a catalytic efficiency ratio only 2-3-fold higher than wild-type PTPα-D2. However, toward RR-src, the PTPα-D1(D382E) mutant had an essentially unchanged Km relative to wild-type PTPα-D1, whereas the Vmax increased 200-fold. Despite this, kcat and the catalytic efficiency ratio of this mutant D1 were still 54- and 136-fold higher than those of wild-type D2. The D1 mutant containing alanine rather than an acidic residue in position 382 (D382A) exhibited similar Km values similar to those of the D382E mutant for both substrates but had a further reduced rate of activity (Table 1). The D1(D382A) mutant and wild-type D1 showed no essential difference in Km values for RR-src, although the Km of the mutant for pNPP was about 5-fold higher than that of wild-type D1 (Table 1). The latter contrasts with a report that same mutation in PTPα-D1 has virtually no effect on the Km for pNPP (24). We do not know the reason for this difference, but in our experiments the mutation of Glu-671 to Ala in D2 had a similar effect on the catalytic efficiency of PTPα-D1 and -D2 mutants toward pNPP and RR-src, respectively, consistent with a role of Asp-382 as a general acid in catalysis.

The above results suggest that in the case of pNPP, PTPα-D1 and -D2 can be induced to behave more, but not entirely, like the other catalytic domain (i.e., D1 like D2 and vice versa) by mutation of their different putative general acids to that present in the counterpart domain, but in the case of the peptide substrate RR-src, the D1 and D2 activities are still quite distinct even after mutation. This is especially true for D2, where the kinetics of RR-src dephosphorylation by the wild-type and E671D forms are virtually the same.

Mutation of Valine 536 of PTPα-D2 to Tyrosine—Most active PTPs have a conserved tyrosine near the N terminus of the catalytic domain, which in PTP1B (Tyr-46) interacts with the phenyl ring of the substrate phosphotyrosine moiety (13). To test if the presence of valine in PTPα-D2 accounted for the reduced activity of D2 relative to that of D1, Val-536 was altered to Tyr. This enhanced the kinetics of dephosphorylation...
of both pNPP and RR-s src, with a 3–4-fold increase in turnover numbers ($k_{cat}$) and reduced $K_m$ values intermediate to that of wild-type D2 and D1 (Table I). Overall, this mutation resulted in a 16- and 5-fold increase in the catalytic efficiency ratio of PTPα-D2 toward pNPP and RR-s src, respectively.

**Mutation of Tyrosine 243 of PTPα-D1 To Valine**—To confirm that this conserved tyrosine was important in D1-mediated catalysis, it was mutated to valine, as found in wild-type PTPα-D2. The PTPα-D1 Y243V mutant exhibited an increased $K_m$ for both pNPP and RR-s src, which was equal to or higher than that of wild-type D2 (Table I). Furthermore, the $k_{cat}$ was reduced by about 3- and 490-fold toward pNPP and RR-s src, respectively. The resulting catalytic efficiency ratios of the D1 mutant were thus reduced compared with wild-type D1, very significantly so (2300-fold) in the case of RR-s src dephosphorylation. Nevertheless, they were still higher than the corresponding catalytic efficiency ratios of wild-type D2.

**Kinetic Analyses of PTPα-D1 and -D2 Double Mutants**—Double mutants PTPα-D1 and PTPα-D2 were created to examine the combined effects of these point mutations on the kinetic parameters. Toward both pNPP and RR-s src, the double mutant PTPα-D1 Y243V/D382E displayed higher $K_m$ and lower $V_{max}$ values than those of either of the D1 single mutants (Table I). Compared with wild-type D1, the catalytic efficiency ratios of the double mutant D1 were reduced about 460-fold for pNPP and 150,000-fold for RR-s src. Compared with wild-type D2, the double mutant D1 was a 3-fold less efficient phosphatase toward both substrates. This demonstrates that the N-terminal Tyr-243 and the nature of the putative general acid are both critical factors for substrate dephosphorylation by D1. Alteration of these two residues to those found in D2 results in a corresponding alteration in the kinetic behavior of D1 to closely resemble that of D2.

**Can D2 be altered to display D1-like kinetics?** Analysis of the double mutant D2 (V536YE671D) shows a dramatic increase in activity toward pNPP, which is higher than either of the single D2 mutants and which results in a 3-fold higher turnover number ($k_{cat}$) and a slightly higher catalytic efficiency ratio than even wild-type D1 (Table I). In contrast, although the kinetic parameters of RR-s src dephosphorylation by double mutant D2 are significantly improved relative to the D2 single mutants, they do not approach those of wild-type D1, with a 160-fold lower $k_{cat}$ and a 560-fold lower catalytic efficiency ratio than wild-type D1 (Table I).

### Table I

**Kinetic parameters of activity of WPD and KNRY mutants of PTPα**

The $K_m$ and $V_{max}$ values represent the mean ± S.E. of experiments conducted with at least three independent preparations of purified proteins.

| PTPα | $K_m$ (mM) | $V_{max}$ (nmol/min/mg) | $k_{cat}$ (s⁻¹) | $k_{cat}/K_m$ (10⁶) |
|------|------------|--------------------------|-----------------|---------------------|
| PTPα-D1$^{a}$ | 0.28 | 18872 | 11.64 | 416 |
| PTPα-D1(D382E) | 1.94 | 2276 | 1.40 | 7.22 |
| PTPα-D1(D382A) | 1.47 | 177 | 0.11 | 0.75 |
| PTPα-D1(Y243V) | 2.23 | 6546 | 4.04 | 18.1 |
| PTPα-D1(Y243V/D382E) | 3.12 | 455 | 0.28 | 0.90 |
| PTPα-D2$^{a}$ | 1.68 | 924 | 0.51 | 3.04 |
| PTPα-D2(E671D) | 3.86 | 8802 | 4.84 | 12.5 |
| PTPα-D2(E671A) | 3.81 | 35 | 0.02 | 0.05 |
| PTPα-D2(V536Y) | 0.45 | 3966 | 2.18 | 48.4 |
| PTPα-D2(V536E/E671D) | 0.76 | 62998 | 34.65 | 456 |

| PTPα | $K_m$ (mM) | $V_{max}$ (nmol/min/mg) | $k_{cat}$ (s⁻¹) | $k_{cat}/K_m$ (10⁶) |
|------|------------|--------------------------|-----------------|---------------------|
| PTPα | 0.032 | 8000 | 4.93 | 1494 |
| PTPα | 0.049 | 40 | 0.02 | 4.08 |
| PTPα | 0.039 | 1.50 | 9.25×10⁻⁴ | 0.24 |
| PTPα | 0.009 | 21 | 0.01 | 0.65 |
| PTPα | 0.041 | 21 | 0.01 | 0.65 |
| PTPα | 0.049 | 21 | 0.01 | 0.65 |
| PTPα | 0.133 | 0.667 | 3.67×10⁻⁴ | 0.03 |
| PTPα | 0.133 | 0.833 | 4.58×10⁻⁴ | 0.03 |
| PTPα | 0.008 | 21 | 0.01 | 0.65 |
| PTPα | 0.010 | 21 | 0.01 | 0.65 |

$^{a}$ Values as previously determined in Ref. 19.

$^{b}$ ND, not determined.

In Vivo Substrate Specificity of PTPα-D1 and -D2—We have previously identified p59$fyn$ as an *in vivo* substrate of PTPα (30). To determine whether one or both catalytic domains of PTPα are involved in the cellular dephosphorylation and activation of p59$fyn$, we employed several forms of full-length mutant PTPα, which contained both tandem catalytic domains but with one of these inactivated by point mutation of the essential cysteine residue to a serine residue (PTPα-D1D2S and D1,D2), or which contained only one catalytic domain due to deletion of the other (PTPα-D1 and -D2). Co-expression of wild-type or mutant PTPα together with p59$fyn$ revealed that PTPα-D1D2S and PTPα-D1, which have an active D1 and an inactive or absent D2, dephosphorylated p59$fyn$ to a similar extent as wild-type PTPα (Fig. 3A). Neither PTPα-D1D2 nor PTPα-D2, having an active D2 and an inactive or absent D2, dephosphorylated p59$fyn$ as efficiently as wild-type PTPα.

Activity of PTPα-D1 and -D2 Y/V and D/E Mutants Toward p59$fyn$ in Vivo—The clearly different abilities of D1 and D2 to dephosphorylate p59$fyn$ make this an ideal substrate for testing the *in vivo* effects of the Tyr/Val and Asp/Glu mutations in each domain. Furthermore, the catalytic ability of mutant D1 or D2 can be examined within the context of the PTPα holoenzyme and not as an isolated entity. The activation of p59$fyn$ was assessed as a measure of co-expressed PTPα activity. The single mutation of Asp to Glu in D1 (PTPα-D1(D382E/D2)) abolished p59$fyn$ activation, as did the double mutation of Tyr and Asp (PTPα-D1(Y243V/D382E/D2)) (Fig. 4, lanes 2 and 4). Although D1 (D382E) exhibited reduced but detectable activity in the *in vitro* assays described above, the absence of *in vivo* activity affected by this mutation likely reflects the lower sensitivity of the cellular assay. Nevertheless, this demonstrates that Tyr-243 and Asp-382 are important, if not essential, for D1 catalysis in *vivo*. No activation of p59$fyn$ was observed with the corresponding single or double mutant D2 (PTPα-D1,D2/E671D).
and PTPα-D1-D2(V536Y/E671D)) (Fig. 4, lanes 5 and 7). Thus, the *in vivo* activity of the D2 mutants parallels the lack of *in vitro* activity seen toward the phosphopeptide RR-src rather than the increased *in vitro* D2-phosphatase activity observed with pNPP.

**FIG. 3.** Dephosphorylation and activation of p59<sup>αα</sup> by various forms of PTPα. A, COS-1 cells were transfected with 2 μg of p59<sup>αα</sup>-D1-D2 or 0.5 μg of empty plasmid (neo) or 0.5 μg of wild-type PTPα or mutant PTPα cDNA. Cell lysates were resolved by SDS-polyacrylamide gel electrophoresis and probed with anti-phosphotyrosine and anti-p59<sup>αα</sup> antibodies, and the signals were quantitated by densitometry to determine the phosphotyrosine content per unit of p59<sup>αα</sup>. B, the phosphotyrosine content of p59<sup>αα</sup> was taken as 100%. Bars indicate the mean ± S.E. of three independent experiments, and the error bars indicate the mean ± S.E. C, representative results from one of the experiments described in A. The p59<sup>αα</sup>-immunoprecipitates were assayed for p59<sup>αα</sup> (top panel) and p59<sup>αα</sup>-immunoprecipitates (bottom panel).

**DISCUSSION**

To address the basis of the kinetic differences between PTPα-D1 and PTPα-D2, we have mutated two residues that are predicted to play key roles in catalysis and which differ between the two domains. One of these amino acids is the acidic residue in the putative movable loops, which is an aspartate in the motif WPD of loop 13 (31) of PTPα-D1 and -D2 and a glutamate in the corresponding WPE sequence of PTPα-D2. Mutation to a non-acidic alanine in D1 or D2 resulted in a pronounced reduction in kinetic activity, as characterized by various *in vitro* assays. The other amino acid we mutated is a hydrophobic residue that is a general acid in D1 or D2, and the mutation to a hydrophobic residue in D1 or D2 resulted in a pronounced reduction in kinetic activity. This, together with the three-dimensional structural similarity between the substrate-free forms of PTPα-D1 (31) and PTPα-D2 (11) and also between the PTPα-D1 and -D2 models (Fig. 1B), which were prepared based on the crystal structure of PTP1B complexed with phosphopeptide (13), suggests that both domains share a catalytic mechanism in common with each other and with other PTPs. The other amino acid we mutated is a hydrophobic residue that is a tyrosine (Tyr-243) in the KNRY motif of loop 1 (31) of PTPα-D1 and a valine (Val-536) in the corresponding position of PTPα-D2. In PTP1B, the equivalent tyrosine residue is located at the top of the catalytic cleft and interacts with the main-chain atoms and phenyl ring of phosphotyrosine in the substrate (13). We report here that both the hydrophobic residue and the general acid are key determinants of the catalytic activities of
PTPα-D1 and -D2. However, the contribution of these residues to the efficiency of dephosphorylation is determined in a large part by the nature of the substrate and highlights major differences between D1 and D2, as discussed further below.

Mutation of either the hydrophilic residue (Tyr-243) or of the general acid (Asp-382) in D1 to the corresponding residue present in D2 (Val or Glu, respectively) reduced the catalytic efficiency of D1 significantly toward the in vitro substrates pNPP and RR-src. Neither mutation alone was sufficient to bring this parameter of activity down to the level observed with wild-type D2, and the D1(Y243V) mutant phosphatase was less efficient than the D1(D382E) mutant in dephosphorylating RR-src, whereas the converse was true for pNPP. The dramatic effect of the D1(Y243V) mutation in reducing the efficiency of RR-src dephosphorylation (4 orders of magnitude from wild-type D1), compared with its much lesser effect in reducing the efficiency of pNPP dephosphorylation (1 order of magnitude from wild-type D1), suggests that Tyr-243 is involved not only in interactions with the phenyl ring of the substrate but also with other elements of the phosphotyrosine microenvironment such as adjacent residues of the substrate. This would be consistent with its positioning near the top of the catalytic cleft. The double mutant D1 (Y243V/D382E) was catalytically very comparable to wild-type D2 with both of these substrates, supporting the view that the observed deviation from invariant residues in these positions of D2 could alone be responsible for the relatively poor enzymatic activity of D2. The synergistic reduction in catalytic activity observed with the double mutant D1 indicates that the orientation of phosphotyrosine in the binding pocket through its interaction with the hydrophilic residue at position 243 may position phosphotyrosine for protonation by the general acid, be it aspartate or, less optimally, glutamate. The substitution of valine for Tyr-243, where valine lacks the bulkier aromatic ring of tyrosine, may eliminate or reduce the interaction with phosphotyrosine, which positions the latter for ready protonation. In accord with the mutant D1 activities in vitro, the in vivo dephosphorylation of p56 

RR-src dephosphorylation (4 orders of magnitude from wild-type D2), and the D1(Y243V) mutant phosphatase was less efficient toward pNPP and RR-src. Neither mutation alone was sufficient to effect a D1-like behavior of double mutant D2 on pNPP and the tyrosyl peptides. More importantly, in stark contrast to the latter for ready protonation. In accord with the mutant D1 activities in vitro, the in vivo dephosphorylation of p56

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