The YRD Motif Is a Major Determinant of Substrate and Inhibitor Specificity in T-cell Protein-tyrosine Phosphatase*

Received for publication, December 26, 2000, and in revised form, March 28, 2001
Published, JBC Papers in Press, May 14, 2001, DOI 10.1074/jbc.M011697200

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We have studied T-cell protein-tyrosine phosphatase (TCPTP) as a model phosphatase in an attempt to unravel amino acid residues that may influence the design of specific inhibitors. Residues 48–50, termed the YRD motif, a region that is found in protein-tyrosine phosphatases, but absent in dual-specificity phosphatases was targeted. YRD derivatives of TCPTP were characterized by steady-state kinetics and by inhibition studies with BzN-EJ-J-amide, a potent inhibitor of TCPTP. Substitution of Asp50 to alanine or Arg49 to lysine, methionine, or alanine significantly affected substrate hydrolysis and led to a substantial decrease in affinity for BzN-EJ-J-amide. The influence of residue 49 on substrate/inhibitor selectivity was further investigated by comparing substrate amino acid preferences of TCPTP and its R49K derivative by affinity selection coupled with mass spectrometry. The greatest effect on selectivity was observed on the residue that precedes the phosphorylated tyrosine. Unlike wild-type TCPTP, the R49K derivative preferred tyrosine to aspartic or glutamic acid. BzN-EJJ-amide which retains the preferred specificity requirements of TCPTP and PTP1B was equipotent on both enzymes but greater than 30-fold selective over other phosphatases. These results suggest that Arg49 and Asp50 may be targeted for the design of potent and selective inhibitors of TCPTP and PTP1B.

Protein-tyrosine phosphatases (PTPs)1 are enzymes that remove phosphate groups from specific tyrosine residues on protein substrates in vivo (for review, see Refs. 1–3). A number of organo-phosphates may, however, serve as substrates for the enzymes in vitro. The enzymes are classified as PTPs on the basis of a unique sequence of amino acids ((I/V)HCX(R/T)) that is found in their active sites and serves to provide a binding site for the phosphorylated tyrosine residue (4). The invariant cysteine residue in the signature motif provides the active site nucleophile for the hydrolysis of the tyrosine phosphate; a mechanism that proceeds through an enzyme-substrate intermediate (5). PTPs were originally not appreciated as attractive targets for therapeutic intervention of certain diseased states since the enzymes were generally thought of as acting in opposition to protein-tyrosine kinases with little specificity. Recently, however, this notion of PTPs is beginning to change with some illuminating studies in cell-based models (6, 7). Accumulating evidence on PTPs suggest that these enzymes are involved in the regulation of specific signaling pathways in the cell and thus their inhibition may not have undesirable pleiotropic effects. For example, the phenotype of PTP1B in mice highlights this phosphatase’s principal role in insulin signaling and the maintenance of glucose homeostasis with no overt increased tumor formation (8, 9). Similar specific roles have been identified for CD45 in thymocyte development and B-cell maturation (10, 11) and for SHP-1 in autoimmune (12, 13). Such studies have provided the impetus for understanding the specificity determinants of the enzymes to allow for their selective inhibition.

It has been estimated that there are ~100 PTPs in the human genome (14). The large number of PTPs in the human genome coupled with their specific regulation of signaling pathways requires that inhibitors targeted toward any one PTP must not only be highly specific but selective as well. It is therefore imperative that structural features that govern the specific interaction of substrates and inhibitors be investigated and understood. We decided to study TCPTP as a model phosphatase in an attempt to unravel specificity determinants in this phosphatase. TCPTP is a ubiquitous enzyme whose substrate in vivo remains unknown. Mice lacking the gene that encodes for TCPTP develop normally in utero but die by 5 weeks of age from defects in hematopoiesis and immune function (15). TCPTP shows a remarkable similarity to PTP1B (see Fig. 1) but it is quite different from most of the other phosphatases in the data base. Results from studies on TCPTP could therefore be compared with those obtained previously for the closely related PTP1B, one of the well studied PTPs. By contrasting results to other phosphatases that show little homology to TCPTP important insights on specificity requirements may thus be gleaned from such studies.

The crystal structure of a catalytically inactive mutant (Cys215 → Ser) of PTP1B in complex with a peptide substrate revealed that residues 48–50 (TCPTP numbering) play important roles in peptide substrate binding (16). Tyr46 interacts with the aromatic ring of the phosphorylated tyrosine residue (Tyr(P)) in the substrate via π–π interactions whereas Arg47 and Asp48 form specific hydrogen bonds with the substrate to orient it in the active site. Site-directed mutagenesis of these implicated residues have corroborated results from the struc-
tural studies (17). Because of the high similarity between TCPTP and PTP1B at the amino acid level this region of the enzyme was targeted. In this report, we present results of our analysis on the influence of this region on substrate and inhibitor binding in TCPTP. Implications of our results for the design of potent and selective inhibitors for this phosphatase and potentially others in the family are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**

HiTrap™ Blue and HiTrap™ Q were obtained from Amersham Pharmacia Biotech. Anti-Flag-agarose and Anti-Flag mAb were purchased from Sigma. The Flag peptide was custom synthesized by Research Genetics. Super Signal west pico chemiluminescent immunoblotting kit was procured from Pierce.

**Methods**

**Cloning**—Full-length human TCPTP was cloned from expressed sequence tags obtained from IMAGE consortium following established methods (18). The catalytic domain (residues 1–281) was subcloned into the EcoRI and SalI unique sites of the pFLAG-2 expression vector by amplifying the appropriate sequences by polymerase chain reaction methodology. Construction of the full-length clone and catalytic domain derivative was confirmed by DNA sequencing on an ABI 373 DNA sequencer (Applied Biosystems) and analyzed by the software application package Sequencer 4.0.5 (Gene Code Corp.).

**Mutagenesis and Protein Expression**—A plasmid that expresses the isolated catalytic domain of human TCPTP, pFlag2-hTCPTP-(1–281), was used as template DNA for the mutagenesis. Construction of the expression vector has been described above (see cloning). The Chameleon™ double-stranded mutagenesis kit (Stratagene) was employed for all site-directed mutagenesis by following the manufacturers instructions. Oligonucleotides used for the site-directed mutagenesis were custom synthesized by Research Genetics (Huntsville, AL). Codons specifying the targeted amino acid changes were incorporated into the mutant oligonucleotide primers as follows (the codon changes that were specifying the targeted amino acid changes were incorporated into the all site-directed mutagenesis by following the manufacturers instructions).

**Protein Purification**—Bacterially expressed proteins were separated bacterially expressed proteins were transferred onto nitrocellulose (OD) of 0.7 at 600 nm in Luria Bertani broth supplemented with 50 mM NaCl, 20% (v/v) glycerol at a pH of 7.5 for storage. All purified proteins were dialyzed into 20 mM Tris-HCl, 0.1 mM EDTA, 5 mM dithiothreitol, 150 mM NaCl, 20% (v/v) glycerol at a pH of 7.5 for storage. All purification steps were carried out at 4 °C or on ice.

An alternate protocol was employed for the purification of the R49A, R49K, and R49M mutant derivatives. The supernatant from the cell lysate was obtained as described above and applied to an anti-Flag mAb affinity column (Sigma). The manufacturer's instructions were followed for the anti-Flag affinity purification step. The appropriate protein fractions were pooled and applied to a Sepharose Q anion exchange column. This and subsequent steps in the purification scheme were performed as described above. A 40 and 50% loss in specific activity was observed for the R49M and R49K derivatives, respectively, following a year of storage at ~80 °C.

**Kinetic Assays**—The catalytic activities and kinetic parameters of all proteins were determined spectrophotometrically by following the hydrolysis of fluorescein diphosphate (FDP) at 450 nm and nitrophenylphosphate (pNPP) at 405 nm as described previously (19). Hydrolysis of DADEpYL was monitored by fluorescence as described by Zhang et al. (20) by excitation at 280 nm and emission at 305 nm. The assays were performed at 25 °C in a buffer that consisted of 50 mM BisTris buffer, 5 mM N,N'-dimethyl-bis(mercaptoacetyl)hydrazine, 2 mM EDTA, 5% (v/v) dimethyl sulfoxide, and 2% glycerol at a pH of 6.3. N,N'-Dimethyl-bis(mercaptoacetyl)hydrazine was excluded from the assay buffer when hydrolysis of DADEpYL was being monitored as it interfered with the assay. Kinetic parameters were obtained by fitting reaction rates data to the Michaelis-Menten equation with the aid of the nonlinear regression analysis package Graphit 4.0.10 (Erithacus Software Inc.).

Inhibition constants of compounds were determined by fitting the rates determined in the presence and absence of inhibitors to the rate equation for competitive inhibition by nonlinear regression analysis using Graphit. The data were also replotted by the method of Dixon (21) for confirmatory purposes. Four different inhibitor concentrations at five different fixed concentrations of substrate (FDP) were analyzed to obtain the family of curves.

**Results**

**LC-MS Analysis of Affinity Selected Peptides**—The affinity selection protocol for peptide analogues has been described previously (24). Following affinity selection, peptides were directly analyzed using LC-MS. All experiments were carried out using a Waters Alliance 2690 high performance liquid chromatography interfaced to a Micromass Quattro LC triple quadrupole mass spectrometer. Samples (25 μl) were injected onto a Phenomenex Luna 4.6 × 50-mm C18 (5 μm) column operated at 1 ml/min. The flow was split such that 200 μl/min went to the mass spectrometer and the remainder to waste. The mobile phase consisted of: A, 12.5 mM triethylamine adjusted to pH 9.8 with acetic acid; B, 5% (v/v) dimethyl sulfoxide, 0.1% (v/v) Triton X-100, and 2% (v/v) glycerol at a pH of 6.3 and a temperature of 25 °C. The kinetic analyses were performed on a Varian Eclipse™ fluorometer equipped with a Peltier temperature control. The synthesis of the peptide inhibitor has been reported previously (23).

**RESULTS**

**PTPs Differ within the YXB Motif**—The crystal structure of an inactive mutant of PTP1B (carrying a C215S substitution) in complex with a phosphotyrosine peptide substrate, DADEpYL-NH₂ revealed that the loop spanning residues 48–50 (TCPTP numbering adopted) is particularly important for substrate binding (16). Shown in Fig. 1 is the region spanning this triad of amino acids and the percent identity regarding the relatedness of other PTPs to PTP1B by amino acid sequence alignment of their catalytic domains. The sequence
Specificity Determinants in TCPTP

Influence of Arg 49 and Asp 50 on TCPTP Inhibition—Specificity Determinants in TCPTP

Characterization of Amino Acid Substitutions on Arg49 and Asp50—To evaluate the influence of the YXB motif on substrate and inhibitor binding in TCPTP, we produced amino acid substitutions at positions 49 and 50. We replaced independently Arg49 to lysine, methionine, and alanine; Asp50 was substituted with alanine and asparagine. The resulting mutant proteins were purified to homogeneity and characterized kinetically (Table I). The Km and kcat values were comparable to the wild-type protein indicating that the presence of the negatively charged carboxyl group was not crucial for substrate binding or turnover. Substitution of the aspartic acid with alanine (D50A), however, reduced the Km for FDP by almost 3-fold without much change in the turnover of the enzyme. It seems possible that the size of the amino acid at position 50 rather than the negative charge per se may be the crucial feature for binding of FDP. All the amino acid substitutions at position 49 resulted in a comparable Km value for FDP. Substituting the native arginine with a conservative lysine (R49K) resulted in a 3-fold increase in Km while essentially maintaining the turnover rate. The R49M mutant protein also showed about a 3-fold increase in Km and about a 2-fold reduction in kcat when compared with the wild-type enzyme. The R49A substitution also resulted in close to a 3-fold increase in Km with only a minor change in kcat.

The effect of the amino acid replacements on hydrolysis of a peptide substrate, DADEpYL, and pNPP are presented in Tables I, II, and III, respectively.

The replacement of Asp50 with Asn did not alter appreciably the kinetic parameters of the resulting mutant, D50N, protein (Table I). The Km and kcat values were comparable to the wild-type protein indicating that the presence of the negatively charged carboxyl group was not crucial for substrate binding or turnover. Substitution of the aspartic acid with alanine (D50A), however, reduced the Km for FDP by almost 3-fold without much change in the turnover of the enzyme. It seems possible that the size of the amino acid at position 50 rather than the negative charge per se may be the crucial feature for binding of FDP. All the amino acid substitutions at position 49 resulted in an increase in the Km value for FDP. Substituting the native arginine with a conservative lysine (R49K) resulted in a 3-fold increase in Km while essentially maintaining the turnover rate. The R49M mutant protein also showed about a 3-fold increase in Km and about a 2-fold reduction in kcat when compared with the wild-type enzyme. The R49A substitution also resulted in close to a 3-fold increase in Km with only a minor change in kcat.

The effect of the amino acid replacements on hydrolysis of a peptide substrate, DADEpYL, presented in Table II. Whereas the D50N substitution resulted in less than a 2-fold reduction in Km with no effect on enzyme turnover, the D50A substitution led to a drastic effect on the kinetic parameters. The substitution resulted in a 16-fold increase in Km and a 10-fold reduction in kcat. The Arg substitutions at position 49 resulted in a 2–3-fold increase in the Km value for the hydrolysis of the peptide substrate. The R49K and R49M replacements also led to a 2–3-fold drop in kcat. In contrast, the R49A mutant showed no appreciable effect on enzyme turnover. Thus, the effect exerted by the arginine substitutions on the Km of the peptide substrate mimicked the trend that was observed with FDP as substrate.

We also investigated the effect of the amino acid substitutions on a much smaller substrate, pNPP (Table III). No appreciable differences were observed for the TCPTP derivatives compared with the wild-type enzyme with respect to the kcat/Km values. Although the D50A derivative also maintained a similar kcat/Km value this was achieved by a compensatory 2-fold reduction in both the Km and kcat values. As the location of residues 49 and 50 on the enzyme are such that an interaction with pNPP is not expected, we conclude that the amino acid changes did not significantly alter the structural integrity of the active sites in the resulting enzymes (perhaps with the exception of D50A). The results suggest that Arg49 may be important for binding of FDP and other substrates to TCPTP. The observation that the Km values obtained with the alanine-substituted protein is similar to that of both the lysine- and methionine-substituted proteins argues against a significant hydrophobic interaction that involves the alkyi side chains of these residues and the substrate.

Influence of Arg49 and Asp50 on TCPTP Inhibition—The differential effects of the arginine and lysine substitutions at
position 49 on FDP and DADEpYL but not on pNPP was intriguing particularly because this residue exists in several PTPs (Fig. 1). We therefore examined further the potential role of Arg⁴⁹ and Asp⁵⁰ on selective inhibition of this enzyme. We investigated the effect of the amino acid changes on the affinity of a potent peptide inhibitor (BzN-EJJ-amide) of TCPTP (Fig. 2a). A derivative of this inhibitor lacking the N-terminal benzoyl group has previously been observed to be very potent on PTP1B (23). As illustrated in the Henderson plot (Fig. 2b), BzN-EJJ-amide is a very potent inhibitor of TCPTP that accurately estimates the concentration of the enzyme (7 nM, the intercept on the ordinate axis) used in the assay. The replot of the slopes (inset) against the concentration of substrate used in the analysis shows that the inhibitor is competitive with an estimated $K_i$ of 1 nM and an $IC_{50}$ of about 4 nM (data not shown) using FDP as substrate. To our knowledge, this compound represents the most potent compound described for a protein-tyrosine phosphatase.

The effect of the amino acid substitutions on the inhibitory potency of BzN-EJJ-amide is presented in Table IV. Substituting Arg⁴⁹ with alanine resulted in a greater than 100-fold decrease in binding affinity of the peptide inhibitor; all the substitutions acted similarly. Thus, we conclude that the interaction between BzN-EJJ-amide and TCPTP appears to involve the guanidinium group of Arg⁴⁹ without much contribution from the alkyl side chain.

Subsite Amino Acid Preferences in TCPTP—Substituting Asp⁵⁰ with alanine resulted in a greater than 100-fold decrease in inhibitory potency of BzN-EJJ-amide clearly establishing the importance of this residue in achieving inhibitor potency.

### TABLE II

**Kinetic parameters for hydrolysis of DADEpYL by wild-type TCPTP and substituted derivatives**

| Kinetic parameters | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
|-------------------|------|---------|---------------|
| Wild-type         | 0.6 ± 0.1 | 21 ± 2 | 34.7 ± 5     |
| D50N              | 1.0 ± 0.1 | 21 ± 1 | 20.5 ± 3     |
| D50A              | 9.8 ± 1.9 | 2 ± 0.3 | 0.2 ± 0.07   |
| R49K              | 1.6 ± 0.3 | 9 ± 1   | 5.4 ± 1      |
| R49M              | 2.1 ± 0.2 | 7 ± 1   | 3.2 ± 0.6    |
| R49A              | 1.9 ± 0.3 | 17 ± 1  | 8.9 ± 1.9    |

### TABLE III

**Kinetic parameters for hydrolysis of pNPP by wild-type TCPTP and substituted derivatives**

| Kinetic parameters | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
|-------------------|------|---------|---------------|
| Wild-type         | 0.80 ± 0.25 | 20 ± 3 | 2.5 ± 1      |
| D50N              | 0.88 ± 0.32 | 21 ± 2 | 2.4 ± 1      |
| D50A              | 0.40 ± 0.13 | 12 ± 1 | 3.0 ± 1      |
| R49K              | 0.79 ± 0.08 | 25 ± 2 | 3.2 ± 0.4    |
| R49M              | 0.75 ± 0.06 | 18 ± 1 | 2.3 ± 0.1    |
| R49A              | 0.95 ± 0.03 | 24 ± 2 | 2.5 ± 0.3    |

### TABLE IV

**Inhibitor potency**

| Inhibitor potency | $IC_{50}$ | $K_i$ |
|-------------------|----------|------|
| Wild-type         | 4 ± 2    | 1 ± 0.3 |
| D50N              | 5 ± 2    | 3 ± 1  |
| D50A              | 50 ± 70  | 243 ± 30 |
| R49K              | 42 ± 6   | 24 ± 3 |
| R49M              | 53 ± 10  | 20 ± 2 |
| R49A              | 49 ± 3   | 23 ± 8 |
substrate. Each of the four positions was analyzed separately by using a library that introduced the 19 different amino acids into that position alone. For example, peptide library $X_4$, DXDEF$_4$PmpL-NH$_2$, has all 19 amino acids independently present at the second position indicated with $X$ in the context of the other invariant amino acids that constitute the substrate analogue. In order to prevent hydrolysis of the phosphopeptide during the analysis, the phosphotyrosine (Tyr(P)) residue was substituted with the non-hydrolyzable difluoro-(phosphonoo)arylphosphorylanine (P$_2$Pmp) moiety.

Subsite preferences were determined by incubating both the wild-type enzyme and R49K derivative separately with the peptide libraries and subsequently analyzing the bound peptide by LC-MS (see “Methods” for details) to determine subsite preferences. Fig. 3 shows the results obtained with the $X_1$ peptide library where position 1 in the substrate was substituted with the different amino acids (XADEF$_2$PmpL-NH$_2$). In this library, no significant difference(s) in amino acid preferences were observed between the wild-type and the R49K proteins. The preferred amino acids at position 1 by both proteins were Asp and Glu. There also appeared to be a selection against the basic amino acids: lysine and arginine. Thus, the R49K substitution does not influence amino acid choice at the $X_1$ position in the substrate analogue.

Next, we investigated the preference(s) for amino acids at position 2 with the $X_2$ peptide library, i.e. DXDEF$_2$PmpL, where $X$ represents any amino acid with the exception of cysteine. As in the case of the $X_1$ library, the amino acids preferentially selected by both proteins at this position were Asp followed by Glu (see Fig. 4). There was a tendency to select against the basic amino acids (arginine, lysine, and histidine) at this position as well. No significant differences were observed between the wild-type enzyme and the R49K derivative. Hence, it appears that the arginine to lysine substitution does not influence substrate specificity with regard to amino acid preferences at the $X_1$ position in the substrate analogue.

An investigation into the subsite requirements for position $X_4$ of the substrate revealed a similar predisposition toward acidic amino acids for both the wild-type and R49K enzymes (see Fig. 5). The preference for acidic amino acids appeared to be higher in the wild-type enzyme than in the R49K mutant; the trend, however, was similar in both cases. As in the previous two positions, there appeared to be a selection against the basic amino acids at position $X_4$.

The most differences between the two proteins were observed with the $X_4$ library representing the amino acid that precedes the Tyr that is dephosphorylated by the enzyme (Fig. 6). As in the previous cases, the wild-type enzyme showed a high preference for Asp and Glu and a lower preference for the basic amino acids. The wild-type enzyme also showed a bias toward the more hydrophobic/aromatic amino acids. In contrast, the R49K derivative preferred the more aromatic amino acids at position 4 over the acidic residues. Both proteins showed no tendency toward either Gly or Pro. There was also a lesser preference for small amino acids or those branched on the $\beta$-carbon atom at $X_4$. Hence, replacing Arg with a conservative lysine resulted in profound changes in substrate/inhibitor specificity of the resulting protein. We conclude from these results that the type of charge rather than the charge per se (at residue 49) is apparently a critical determinant of selectivity.

Selective Peptide Inhibitors of TCPTP—The MS data on the wild-type and R49K enzymes provided clues for subsite preferences in TCPTP inhibitor design that we wished to test directly on the enzymes. Results from Fig. 6 suggested that the incorporation of Asp at $X_4$ would yield a good inhibitor for the wild-type enzyme but a relatively poor inhibitor for the mutant enzyme. Finally, a peptide inhibitor with Glu rather than Asp at $X_4$ and $X_5$ should have approximately equivalent $K_i$ values on the wild-type enzyme but not on the R49K mutant derivative (Figs. 5 and 6). We therefore determined inhibition constants ($K_i$ values) for three peptide analogues on both enzymes to test the predictions. The inhibition constants of the peptide inhibitors are presented in Table V.

The kinetic plots indicated that the inhibitors tested were all competitive toward pNPP for both enzymes. Similar results are obtained with FDP as substrate (data not shown). As suggested by the MS data the presence of an Asp at $X_4$ resulted in a poorer inhibitor for the R49K derivative. A 29-fold drop in inhibitor potency was observed compared with that obtained for the wild-type enzyme. When the Asp residue in peptide inhibitor-1 was substituted with a Tyr (inhibitor-2) a greater than 10-fold improvement in binding affinity on TCPTP-R49K was observed as predicted by the MS data. Also consistent with the data for subsite preferences, the presence of Glu rather Asp (inhibitor-3) at $X_4$ and $X_5$ results in a 4 -times more potency on the R49K mutant enzyme. The wild-type enzyme exhibited equivalent $K_i$ values whether the $X_4$ amino acid was an Asp or Tyr (inhibitors 1 and 2). Thus, the change in preference for aromatic amino acids at $X_4$ observed with the R49K protein (Fig. 6) appears to result from its inability to interact with acidic amino acids unlike the wild-type enzyme. Finally, equivalent $K_i$ values were observed for the wild-type protein whether an Asp or Glu is retained at $X_4$ and/or $X_5$ (cf. 2 versus 3, Table V).

The design of BzN-EJJ-amide incorporated the preferred acidic amino acid before the tyrosine that is dephosphorylated. Hence, it was of interest to evaluate the selectivity of the inhibitor on several phosphatases. The inhibitor was equipotent on the highly related TCPTP and PTP1B but showed a greater than 30-fold selectivity over the other phosphatases tested (Table VI). The inhibitor was also quite selective over PTP-meg1, a phosphatase that also retains the YRD motif but appears to be structurally quite distinct from PTP1B and TCPTP based on amino acid homology in the catalytic domain (see Fig. 1). The highest selectivity for a tyrosine-specific phosphatase of 3 orders of magnitude was observed over LAR. The selectivity over the dual-specificity phosphatase, Cdc25, was even much higher at approximately 4 orders of magnitude. Because the conservative substitution of Arg$^{49}$ to lysine alone
in TCPTP led to a 10–20-fold reduction in potency of BzN-EJJ-amide, we conclude that the YRD motif is necessary but not sufficient to account for the selectivity observed over the phosphatases.

**DISCUSSION**

Owing to the involvement of distinct PTPs in several cellular signaling processes they have increasingly become attractive targets for the design of drugs to intervene in certain disease states such as type-2 diabetes (8). As PTPs are highly related and employ the same chemistry to catalyze their reactions, the need to unravel determinants of substrate and inhibitor selectivity cannot be overemphasized. Previously, we reported on the possibility of synthesizing specific active-site directed, reversible inhibitors for this family of enzymes (23). In this report, we have studied TCPTP as a model phosphatase in an attempt to delineate structural features that may influence selectivity in this phosphatase. To this end, we targeted residues 48–50 (the YXB motif) on the basis of structural data and previous studies on PTP1B (16, 17). As TCPTP is identical to PTP1B within this region (Fig. 1) and shows similar substrate specificity, we presumed that residues 48–50 would play important roles in substrate/inhibitor binding. Interestingly, a similar motif is not found in the closely related dual-specificity phosphatases that hydrolyze both phosphorylated tyrosine and serines/threonine residues. Thus, the motif represents potentially a distinct structural feature for PTPs that could be exploited for inhibitor selectivity.

**Tyr**

The first member of the YXB triad is highly conserved among PTPs as it interacts with the phosphotyrosine moiety of substrates (16). The influence of this residue on selectivity

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**TABLE V**

| Peptide inhibitor, $X_1X_2X_3F_2$PlpL-NH$_2$ | Inhibition constant, $K_i$ (nM) |
|---------------------------------------------|-----------------------------|
| Wild-type TCPTP                            | 1. DADD(F$_2$Pmp)L-NH$_2$  | 67 ± 8 |
| R49K derivative                             | 2. DADY(F$_2$Pmp)L-NH$_2$  | 74 ± 3 |
|                                             | 3. DAEE(F$_2$Pmp)L-NH$_2$  | 61 ± 13|

**TABLE VI**

| PTP            | IC$_{50}$ (μM) |
|----------------|---------------|
| TCPTP          | 0.006         |
| PTP-1B         | 0.006         |
| PTPβ           | 0.2           |
| SHP-2          | 0.4           |
| SHP-1          | 0.5           |
| PTP-meg1       | 1.9           |
| CD45           | 2.1           |
| LAR            | 6.3           |
| Cdc25          | >50           |
within the family of enzymes is therefore expected to be minimal based on mechanistic considerations. Hence, this residue was not pursued as a selectivity candidate. Substantial variability is, however, observed at position 49 where lysine predominates and arginine is found in TCPTP and PTP1B. The substitutions introduced at position 49 were selected to evaluate the contributions of the positive charge and alkyl side chain of the Arg residue. The R49K-, R49M-, and R49A-substituted proteins all had about a 3-fold increase in the $K_m$ value for FDP and DADEpYL hydrolysis. The Arg-substituted proteins also showed some decrease in the $k_{cat}/K_m$ for the hydrolysis of FDP compared with the wild-type protein. Residue 49 is not expected to influence hydrolysis of the E-P intermediate assuming TCPTP folds similarly to the highly homologous PTP1B. This is because in the crystal structure of PTP1B$^{215S}$ bound to a peptide substrate or to phosphotyrosine this residue is relatively far removed from the phenylphosphate reaction center. Because the Arg substitutions do not affect pNPP hydrolysis the amino acid changes apparently do not significantly affect the active site conformation. Hence, it is presently not clear how the Arg substitutions influence enzyme turnover in regards to hydrolysis of FDP and DADEpYL.

The amino acid residue at position 50 in PTPs is largely restricted to Asp or Asn. We substituted Asp$^{50}$ with Asn and Ala to assess the contribution of the carboxyl group to inhibitor/substrate specificity. The D50N mutant protein catalyzed the hydrolysis of FDP with similar kinetics as the wild-type protein. Eliminating the carboxyl group to the methyl, however, resulted in a protein (D50A) that hydrolyzed FDP with a 3-fold higher efficiency than the wild type enzyme. The effect on $k_{cat}/K_m$ was largely due to a lower $K_m$ value. In contrast, substitution of Arg$^{49}$ with alanine, methionine, and lysine all led to an increase in $K_m$ for FDP hydrolysis. Our results suggest a likely interaction between the second phosphate moiety of FDP and the loop spanning Arg$^{49}$ and Asp$^{50}$. It is conceivable that the second phosphate group in FDP interacts with the guanidinium moiety of Arg$^{49}$ as reflected in the increase in the $K_m$ value for the Arg$^{49}$-substituted proteins. The improved affinity with the much smaller D50A substitution may reflect a relief of steric hindrance that facilitates a more productive binding. The similar $K_m$ values for FDP hydrolysis observed with the lysine, methionine, and alanine mutants may reflect the inability of the terminal amino group of lysine to participate in bidentate interactions with a phosphate group. It is of interest to note that the arginine and aspartic acid replacements exhibited no change in the $K_m$ for the hydrolysis of pNPP compared with the wild-type enzyme (Table III). Hence, the effects observed with FDP appears to reflect specifically how this substrate interacts with the enzyme. We are attempting to crystallize and solve the structure of FDP in complex with a PTP to address this issue. In contrast to FDP, the D50A substitution resulted in an enzyme that hydrolyzed DADEpYL 170-fold less efficiently. The effect on $K_m$ may be explained on the basis of the x-ray structure of PTP1B$^{215S}$ in complex with DADEpYL. Asp$^{50}$ (TCPTP numbering) accepts two hydrogen bonds from the backbone amides of the substrate. The Ala unlike the Asn replacement will be incapable of such interactions. The effect on $k_{cat}$ is not clear because residue 50 is not expected to influence the dephosphorylation step for reasons discussed above for residue 49.

In order to evaluate the potential contribution of the YRD motif in TCPTP for the design of selective inhibitors, we examined the effect of Arg$^{49}$ and Asp$^{50}$ substituted proteins on a potent inhibitor of this enzyme, BzN-EJJ-amide is a competitive inhibitor of TCPTP with a $K_i$ of $-1 \text{ nm}$. It does not exhibit slow-binding behavior and binds the enzyme reversibly. The isosteric D50N substitution did not affect the affinity of the inhibitor but the D50A-substituted protein bound the inhibitor $\sim$200-fold less tightly. Hence, we attribute the loss of affinity of the D50A protein for BzN-EJJ-amide to an inability of the alamine side chain to hydrogen-bond to the inhibitor. The loss of $\sim$3.1 kcal/mol (RT$lnK$) binding energy with the D50A substitution will be consistent with this interpretation of the results. These results suggest that inhibitor interactions that involve Asp$^{50}$ may be exploited for remarkable inhibitor potency in TCPTP. In fact, while this work was in progress Iversen et al. (26) reported on inhibitors that exploit the equivalent of this residue in PTP1B for potency. This residue, however, does not appear to represent a suitable target for inhibitor selectivity because among PTPs amino acid preferences at position 50 is largely restricted to Asp or Asn. In contrast to amino acid preferences at position 50, several PTPs differ greatly in the residue at position 49 making this residue an attractive candidate for inhibitor selectivity. It is interesting that the amino acid substitutions that were introduced at position 49 resulted in substantial losses in affinity of BzN-EJJ-amide for the proteins, a result which reflected that observed with the peptide analogues (Fig. 6; Table V).

As illustrated in Fig. 2, the inhibitor has two phosphonate groups whose binding orientation remains unclear. It is expected that one of the phosphonate moieties will interact within the PTP-signature motif, IHCSAGIGRS, or primary binding site. How does the second phosphonate group interact with the enzyme? A crystal structure of PTP1B in complex with phosphotyrosine has revealed the presence of a second aryl phosphate-binding site in the enzyme (27). One possibility is that the second phosphonate group may interact at this secondary binding site defined by Arg$^{26}$ and Arg$^{252}$ in TCPTP. Preliminary results, however, suggests that an R26A substitution in TCPTP does not affect the affinity of BzN-EJJ-amide for the enzyme. The results with the mutant proteins appear to suggest an alternative binding mode for this peptide inhibitor. The similar potencies obtained with the three Arg substitutions argue for a binding orientation of the inhibitor where Arg$^{49}$ interacts with the inhibitor potentially via bidentate hydrogen bonding for which lysine would be incapable. We cannot, however, predict with any certainty in the absence of a crystal structure whether Arg$^{49}$ interacts with either the carboxyl and/or phosphonyl groups of BzN-EJJ-amide. Suffice it to say that the interaction of the inhibitor with TCPTP appears to involve Arg$^{49}$ and Asp$^{50}$ in a specific manner.

As residue 49 potentially represents a target for TCPTP selectivity we explored how the conservative R49K change alone may influence subsite amino acid preferences. We have previously reported on the development and validation of the affinity selection approach using PTP1B as our model phosphatase (Ref. 24, see “Methods for Modifications”). A similar approach was taken to map out subsite preferences in wild-type TCPTP and its R49K derivative. The peptide library used for these studies spanned the autophosphorylation site of the epidermal growth factor receptor; a receptor that is apparently dephosphorylated by TCPTP (28). Amino acid preferences observed with the R49K derivative were comparable to the wild-type protein for all positions except for the residue that immediately precedes the phosphotyrosine. The R49K protein interacted preferentially with more aromatic and hydrophobic residues at this position ($X_9$) in contrast to the wild-type enzyme that preferred acidic residues but also bound hydrophobic residues at this position in the substrate analogues (Fig. 6).

The MS results and subsequent inhibition studies indicated that the change in amino acid preference was a result of the R49K derivatives inability to interact effectively with acidic
Specificity Determinants in TCPTP

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Acknowledgments—We express our sincere gratitude to our colleague Dr. Zheng Huang for critical reading of the manuscript. We extend our sincere thanks to Vira Patel for technical assistance. We are also very grateful to Drs. Kathleen Metters and Mike Greiser for continued support.
The YRD Motif Is a Major Determinant of Substrate and Inhibitor Specificity in T-cell Protein-tyrosine Phosphatase
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J. Biol. Chem. 2001, 276:26036-26043.
doi: 10.1074/jbc.M011697200 originally published online May 14, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M011697200

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