Regions of protein-tyrosine phosphatase (PTP) 1B that are distant from the active site yet affect inhibitor binding were identified by a novel library screen. This screen was based on the observation that expression of v-Src in yeast leads to lethality, which can be rescued by the coexpression of PTP1B. However, this rescue is lost when yeast are grown in the presence of PTP1B inhibitors. To identify regions of PTP1B (amino acids 1–400, catalytic domain plus 80-amino acid C-terminal tail) that can affect the binding of the difluoromethyl phosphonate (DFMP) inhibitor 7-bromo-6-difluoromethylphosphonate 3-naphthalenenitrile, a library coexpressing PTP1B inhibitory PTP1B mutants and v-Src was generated, and the ability of yeast to grow in the presence of the inhibitor was evaluated. PTP1B inhibitor-resistant mutations were found to concentrate on helix α7 and its surrounding region, but not in the active site. No resistant amino acid substitutions were found to occur in the C-terminal tail, suggesting that this region has little effect on active-site inhibitor binding. An in-depth characterization of a resistant substitution localizing to region α7 (S295F) revealed that this change minimally affected enzyme catalytic activity, but significantly reduced the potency of a panel of structurally diverse DFMP PTP1B inhibitors. This loss of inhibitor potency was found to be due to the difluoro moiety of these inhibitors because only the difluoro inhibitors were shifted. For example, the inhibitor potency of a monofluorinated or non-fluorinated analog of one of these DFMP inhibitors was only minimally affected. Using this type of library screen, which can scan the nearly full-length PTP1B sequence (catalytic domain and C-terminal tail) for effects on inhibitor binding, we have been able to identify novel regions of PTP1B that specifically affect the binding of DFMP inhibitors.

A traditional approach to understanding the structural interactions between an inhibitor and enzyme is through crystallographic studies of the inhibitor–enzyme complex. Solving the three-dimensional structure of such complexes can identify the key residues that interact with the inhibitor, providing an understanding of the mechanism of inhibition. This is normally accompanied by site-directed mutagenesis of the interacting residues to validate their role in inhibitor binding and to determine the degree to which this interaction contributes to inhibitor potency. Such structures are also important in drug design, as they allow the identification of potential interactions that could improve inhibitor potency using further chemical modifications.

Protein-tyrosine phosphatase (PTP) 1B (EC 3.1.3.48), a potential drug target for the treatment of diabetes and obesity (1, 2), has had a number of published inhibitor–enzyme complexes, and the determinants required for the binding of various inhibitors have been identified. Because these inhibitors all target the active site, the determinants identified are located in the active-site region. These include residues on the PTP loop (Ser216, Ala217, Gly218, Ile219, Gly220, and Arg221), on the WPD loop (Asp181 and Phe182), and on the substrate recognition loop (Lys36, Val49, and Lys120) and for inhibitors interacting with the secondary binding site (Thr205, Arg24, His25, Phe25, and Arg250) (for reviews, see Refs. 3 and 4; see Fig. 4).

Because of the static nature of crystal structures, only direct interactions between inhibitor and enzyme are observed. Because inhibitor/enzyme interactions are quite dynamic, it was of interest to determine whether residues in regions of PTP1B other than the active site could also influence inhibitor binding. A very well documented example of this is the drug-resistant mutations that arise in the human immunodeficiency virus type 1 protease during the treatment of AIDS. These mutations reduce inhibitor affinity, but maintain sufficient substrate affinity for the virus to replicate. Although some of these mutations are located in the active site, there is also an accumulation of mutations in regions distant from the active site (5, 6).

In a similar manner, Colicelli and co-workers (7, 8) have used a yeast expression system to identify drug-resistant mutants for mammalian phosphodiesterases (PDEs). PDE-deficient yeast cells are sensitive to heat shock, but this phenotype can be complemented by expression of mammalian PDEs. Treatment of yeast expressing human PDE4 with specific PDE4 inhibitors such as rolipram abolishes this complementation and reverts the yeast to heat shock sensitivity. A random mutagenesis study was performed using this phenotypic readout to identify residues responsible for PDE4 drug sensitivity (7, 8).

We used a similar approach to identify additional regions of PTP1B that may influence the binding of the difluoromethyl phosphonate (DFMP) inhibitors (9–11). We recently described a yeast screen for PTP1B inhibitors whereby yeast growth is dependent on PTP1B activity (12). The assay is based on the observation that expression of v-Src in
yeast is lethal, but can be rescued by the coexpression of PTP1B. However, this rescue is lost when yeast cells coexpressing v-Src and PTP1B are grown in the presence of specific PTP1B inhibitors. To identify novel determinants of PTP1B inhibitor binding, a yeast library expressing v-Src and random mutants of nearly full-length PTP1B was generated and grown in the presence of the DFMP inhibitor ?-bromo-6-difluoromethylphosphonate 3-naphthalenemine (Inhibitor 1). Inhibitor-resistant colonies were recovered and sequenced, and the regions of PTP1B responsible for inhibitor resistance were identified. Inhibitor-resistant mutations clustered on helices α3, α6, and α7, but not in the active site. One of these mutations was characterized in detail and shown to elicit inhibitor resistance by selecting against the difluoro moiety of the DFMP inhibitor.

MATERIALS AND METHODS

Yeast Strain and Plasmid Constructs—The yeast strain used was YPH499 (MATa ura3-52 lys2-801mbr ade2-101mbr trp1-D63 his3-D200 leu2Δl; Stratagene). p416GAL1 (ATCC 87332) and p415GALL (ATCC 87338) are low copy vectors containing the URA3 marker for growth in uracil-deficient media and the LEU2 marker for growth in leucine-deficient media, respectively. The details of the construction of p416GAL1-PTP1B-(1–320) and p415GALL-v-Src have been described previously (12).

PTP1B-(1–400) was generated from the full-length construct (amino acids 1–435) by PCR using primers 5′-GAATTCATGGGATG-3′ and 5′-GTCGACCATGCTAGTTCTCGTCTTCC-3′ to introduce EcoRI and Sall restriction sites and the following amplification program: 94 °C for 4 min and 25 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1.5 min, followed by 72 °C for 10 min. The resulting PCR product was inserted in the pCR®2.1-TOPO vector (TA Cloning®, Invitrogen). The PTP1B fragment was digested from the pCR®2.1 vector using EcoRI and Sall, cloned into p416GAL1, and sequenced. The S295F PTP1B-(1–320) mutant from the library screen was subcloned from p416GAL1 into pFLAG2 (Kodak) using EcoRI and Sall.

Inhibitors—The PTP1B allosteric inhibitor 3-(3,5-dibromo-4-hydroxybenzyl)-2-ethylbenzofuran-6-sulfonic acid-4-(thiazol-2-ylsulfanyl)phenyl-amide was obtained from Calbiochem (13). All other inhibitors were synthesized at Merck Frosst Canada Ltd., and the methods of synthesis have been described (10, 11, 14).

Generation of the PTP1B Degenerate Library—A degenerate PCR was performed using a low fidelity polymerase (GeneMorph, Stratagene), 565 ng of p416GAL1-PTP1B-(1–400), and 100 ng of 5′-GATATATACCTTATACCTTTAC-3′ as forward primer and 5′-CTTCTTCTTCGGTTAGAGCGGATGCTTC-3′ as reverse primer with the following program: 94 °C for 4 min and 25 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1.5 min, followed by 72 °C for 10 min. These primers were designed to amplify a product starting 83 bp upstream of the PTP1B marker for growth in uracil-deficient media and the polylinker fragment and grown in the presence of the DFMP inhibitor ?-bromo-6-difluoromethylphosphonate 3-naphthalenemine (Inhibitor 1). The primer product was sequenced, and the mutation rate obtained was 8.5 clones were sequenced, and the mutation rate obtained was 150 bp.

Library Transformation—YPH499 cells were electroporated (15) with plasmids p415GALL or p415GALL-v-Src. After 2 days of growth, 50 ml of leucine-deficient medium was inoculated with the resulting transformant for overnight growth. The resulting culture of yeast transformed with p415GALL-v-Src was re-transformed with the degenerate library using the lithium acetate method along with 1 μg of p416GAL1 vector linearized with EcoRI and Sall, dephosphorylated, and plated on leucine- and uracil-deficient plates with 2% galactose and 0.075% glucose (16). An aliquot of the transformation was also plated on leucine- and uracil-deficient plates with 2% glucose to monitor the number of transformants obtained.

Inhibitor Resistance Assay—The colonies resulting from the double transformation were picked after 3, 4, or 5 days of growth on agar plates and inoculated (~0.2 μl) in 50 μl of 15% glycerol in the 60 center wells of a round bottom 96-well plate. 10 μl of this suspension was transferred to the center wells of a flat bottom 96-well plate containing 190 μl of leucine- and uracil-deficient medium with 2% galactose and 0.75 mM Inhibitor 1. The wells were overlaid with mineral oil and incubated at 30 °C, and the absorbance at 600 nm was read periodically (12). The leftover glycerol suspension was stored at ~80 °C.

Plasmid Retrieval—Yeast plasmids from the frozen glycerol stock were retrieved from a 3-ml overnight culture. Briefly, the culture was treated for 1 h with 50 units of lyticase (Clontech), vortexed for 5 min with 250 μl of 400–500-μm glass beads, and purified with QiAprep spin minipreps (Qiagen Inc.). Larger yields of DNA used for sequencing were obtained by transforming MAX Efficiency® DH5α™ Escherichia coli cells (Invitrogen) with the material obtained from the yeast extraction and re-isolating the plasmid. The resulting plasmids were digested with EcoRI and Sall to confirm the presence of a library fragment and sequenced.

Enzyme Kinetics—Wild-type PTP1B and the S295F mutant were expressed and purified from E. coli BL21 cells, and enzyme activities were assayed with p-nitrophenyl phosphate (pNPP), 3,6-fluorescein diphasphate (FDP), or difluoromethylbenzofuran (DifMUP) in 50 mM BisTris, 2 mM EDTA, 2% glycerol, 0.01% Triton, and 5 mM N,N'-dimethyl{(mercaptopoctaoyl)hydroxide} (pH 6.3) as described previously (17, 18). Product formation was monitored spectrophotically at 405 nm for pNPP and at 450 nm for FDP and by fluorescence at 450 nm from 360 nm excitation for DifMUP. Michaelis-Menten parameters and turnover numbers were obtained by fitting the initial rates to the Michaelis-Menten equation using the nonlinear regression analysis package GraFit Version 4.0.10 (Eraticus Software Inc.).

IC50 values were obtained from the initial rates of the reaction of wild-type or mutant PTP1B with the respective Kcat values for DifMUP in the presence of inhibitor, fitted to a four-parameter inhibition curve. Kcat values were obtained following the method of Cornish-Bowden (19) and analyzed using SigmaPlot (SYSTAT). For the allosteric inhibitor, the buffer conditions for the reaction were modified as follows: 50 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol, and 100 mM NaCl as described (13).

Crytalization and Data Collection—Apo-S295F mutant PTP1B crystals were obtained by vapor diffusion in sitting drops at 4 °C by mixing 2 μl of protein (10 mg/ml in 20 mM HEPES, 50 mM NaCl, 1 mM EDTA, and 5 mM N,N'-dimethyl{(mercaptopoctaoyl)hydroxide} (pH 7.0)) and 2 μl of precipitant solution (13–16% polyethylene glycol 3350, 100 mM HEPES, and 200 mM MgCl2 (pH 7.0)). X-ray diffraction data were collected on an ADSC Q4 detector from a single crystal of ~0.1 mm in size. Data to 2.0-Å resolution were collected using an F-RD rotating anode (Rigaku) operating at 50 mA and 90 mA. Data processing, scaling, and merging were done with the software DPP/MOSFLM (20, 21). The crystal was trigonal, with space group P321 and unit cell parameters a = b = 88.33 Å, c = 104.18 Å, α = β = 90.0°, and γ = 120°. Table 1 summarizes the statistics for the data collected.

Structure Solution and Refinement—The crystal was isomorphous with previously reported PTP1B crystals (i.e. Protein Data Bank code 5259).
Yeast Screen for PTP1B Inhibitor Determinants

TABLE 1
Statistics for the data set used to solve the structure of the apo-S295F variant of PTP1B and final statistics for the refined model

| Data collection | Resolution range (Å) | 50.0 to 2.0 (2.1 to 2.0) |
|-----------------|----------------------|---------------------------|
| No. of reflections | 31,482 (4049) |
| % possible | 97.7 (87.7) |
| Redundancy | 10.1 (7.7) |
| l/| | 9.1 (2.2) |
| R<sub>free</sub> | 6.9 (34.4) |

| Refinement statistics | Resolution range (Å) | 30.0 to 2.0 (2.1 to 2.0) |
|-----------------------|----------------------|---------------------------|
| No. of reflections (work)<sup>a</sup> | 29,892 (2478) |
| % possible<sup>a</sup> | 92.7 (78.0) |
| R<sub>free</sub>/R<sub>free</sub> | 20.3/18.3 (25.4/20.6) |
| R.m.s.d. bond length/angle | 0.011/1.45° |
| No. of protein atoms<sup>b</sup> | 2754 |
| No. of solvent atoms | 251 |
| No. of heteroatoms<sup>b</sup> | 5 |

<sup>a</sup> 5% of the reflections were set aside for R<sub>free</sub> calculation.
<sup>b</sup> This includes alternate conformations.
<sup>c</sup> Two magnesium and three chlorine ions have been identified in the structure.

1PTY) (22), and the three-dimensional structure of the S295F mutant was solved by difference Fourier using as an initial model the 1.8-Å structure of the mutant enzyme in complex with phosphotyrosine (Protein Data Bank code 1PTY). Bound ligand, solvent molecules, and protein residues 175–184 (WPD loop) and 285–295 (helix α7) were deleted from the coordinate file. Refinement of the model was carried out by alternating cycles of manual rebuilding of the model in program O (23) and computer-based refinement using CNX, slowly including all available data to 2.0-Å resolution. Table 1 summarizes the statistics for the refined model.

RESULTS

Use of Yeast to Identify Amino Acid Determinants of PTP1B Inhibitor Binding—Recently, we reported on a yeast assay to screen for PTP1B inhibitors (12). The assay is based on the observation that v-Src overexpression in yeast is lethal, but yeast growth can be rescued by coexpression of PTP1B. This rescue is reversed when yeast cells coexpressing v-Src and PTP1B are grown in the presence of PTP1B inhibitors. Using this assay, the approach we took to identify inhibitor determinants was first to generate a library of PTP1B mutants by random mutagenesis using degenerate PCR. Yeast cells expressing v-Src were then transformed with the PTP1B mutants. If the mutation significantly affected PTP1B catalytic activity, the yeast cells did not grow. Those that grew were then cultured in the presence of specific PTP1B inhibitors. For the yeast to grow under these conditions, the PTP1B mutation must not only be catalytically active, but also have a reduced affinity for the inhibitor. Plasmids were then recovered from these resistant clones and sequenced to identify the residue that had been substituted.

The PTP1B inhibitor that was used to screen for inhibitor determinants is shown in Fig. 1. We have shown previously that this inhibitor has no effect on yeast growth even at very high concentrations, but can efficiently inhibit the growth of yeast coexpressing v-Src and PTP1B with an EC<sub>50</sub> of 0.17 mM (12). The binding mode of the DFMP inhibitors in the PTP1B active site is well understood. In fact, the crystal structure of PTP1B complexed with (1,1-difluoro-1-(2-naphthalenyl)methyl)phosphonic acid, a very close analog of Inhibitor 1, has been reported previously (24). We have modeled Inhibitor 1 in the PTP1B active site to illustrate the direct interactions the inhibitor makes with the enzyme (Fig. 1). The DFMP group of Inhibitor 1 engages the phosphatase by extensive hydrogen bonding of the phosphonate with the PTP loop (24, 25). The two fluoride atoms are within van der Waals distance of the phenyl side chain of Phe<sup>182</sup> and are hydrogen-bonded through a water molecule to the side chain nitrogen of Gin<sup>266</sup> and the main chain nitrogen of Phe<sup>182</sup> (26, 27). The remainder of the molecule is situated within a hydrophobic pocket, with the naphthalene ring sandwiched between Tyr<sup>266</sup> and Phe<sup>182</sup> and the nitrile group pointing toward the secondary binding site.

FIGURE 1. Modeling of Inhibitor 1 into the PTP1B active site. The structure of Inhibitor 1 is shown in the inset. The active-site nucleophile Cys<sup>215</sup> in the PTP loop (pink) is shown along with Phe<sup>182</sup> in the WPD loop (green). Inhibitor 1 sits in the active site with the phosphonate (red) interacting with the PTP loop, and the difluoro group is within van der Waals distance of the phenyl side chain of Phe<sup>182</sup>.

FIGURE 2. Growth curves of yeast cultured in the presence of Inhibitor 1. Yeast expressing only PTP1B (○) had growth equivalent to that of the wild-type yeast (12) even in the presence of Inhibitor 1. Similar growth was also observed for the false positive (false +ve) clone 93 (■). No growth was observed for yeast coexpressing v-Src and wild-type PTP1B (□) in the presence of Inhibitor 1 or for yeast expressing v-Src (□) (lethal) (12). However, significant growth over the control (yeast expressing v-Src and wild-type PTP1B) was observed for resistant clones coexpressing v-Src and a PTP1B mutant. Resistant clones also displayed a slight delay in growth compared with yeast expressing PTP1B alone. Shown are representative growth curves for three resistant clones: 838 (△), 979 (●), and 1036 (□) (see Table 1).
TABLE 2

Nucleotide mutations and associated amino acid changes in inhibitor-resistant clones

| Location            | Clone       | Nucleotides                  | Amino acids                      |
|---------------------|-------------|------------------------------|----------------------------------|
| N terminus of helix α3 | 1204        | G556A                        | E1186K                           |
|                     | 135         | A557C, A1189del              | E1186A, K397R then TRHRHWSTSSHVISYVTLTFTPSPHIRSNKGRS |
|                     | 460         | G424A, A400T, A422C, G556A, C1117T | M114L, I134F, K444T, E166K, R373W |
|                     | 1909        | T595C (T855A)                | S187P                            |
|                     | 722         | C566T                        | A189Y                            |
|                     | 1480        | (C-35A), (C-29T), T810A, A441C, (T528C), C573A, G1069A, G1126T | H60Q, E147D, F191L, A357T, G376W |
| C terminus of helix α6 and turn α6–α7 | 546         | G108T, E828T, G847A, G952T, (A1019G) | K36N, E276D, G283R, E318stop |
|                     | 8           | G850del                      | D284T then LPCRISGRSPFRTWSPHPISPHLPGPHPNESWHTMGN-AGSSQITSG |
|                     | 355         | G846del                      | D284T then LPCRISGRSPFRTWSPHPISPHLPGPHPNESWHTMGN-AGSSQITSG |
| Helix α7            | 754         | (A756G), C857T, C984A, A988G, C1163T, A1199G | M3I, E4K, H208Q, S286F |
|                     | 176         | G97T, G10A, C624G, (C672T), C857T, (G1197A) | S288R then ISGRSPFRTWSPHPISPHLPGPHPNESWHTMGNAG-SSQITSG |
|                     | 742         | C862del                      | Q288stop                          |
|                     | 46          | C862T, (A916G)               | Q288stop                          |
|                     | 362         | (C21T), C868T, (A1044T)      | Q290stop                          |
|                     | 1504        | (G798A), C868T, (C984T)      | W291stop                          |
|                     | 235         | G873A                        | W291stop                          |
|                     | 670         | G872A                        | T291stop                          |
|                     | 404         | A112T, G873A, (A1111T)       | S550R, W291stop                   |
|                     | 979         | T150A, G872A                 | S292E                             |
|                     | 838         | A874G                        | S295F                             |
|                     | 1036        | C884T                        | S299Y1, V375M                     |
|                     | 1422        | C884A, G1123A, (C1185A)      | H2961 then RTWSPHPISPHLPGPHPNESWHTMGNAG-SSQITSG |
|                     | 1117        | C886del                      | A278V, E297K                      |
|                     | 2034        | C833T, G889A                 | K279N, L299M                      |
|                     | 2039        | A65C, G889A                  | D22A, E297K                       |
|                     | 2067        | A837C, G895A                 | K279N, L299M                      |

In addition, although there have been numerous crystal structures of PTP-1B with and without inhibitor, all have been done with the catalytic domain (amino acids 1–320 or 1–298), which excludes the effect of the PTP1B C terminus on inhibitor binding. The full-length PTP1B sequence is 435 amino acids, with the last 35 C-terminal amino acids anchoring PTP1B into the endoplasmic reticulum (26). Therefore, we decided to use PTP1B-(1–400) without the endoplasmic reticulum-anchoring PTP1B into the endoplasmic reticulum (26). Therefore, we decided to use PTP1B-(1–400) without the endoplasmic reticulum-anchoring sequence for the screen.

Identification of Novel PTP1B Inhibitor Determinants—A yeast library (~31,000 transformatons) expressing v-Src and containing random mutants of PTP1B-(1–400) was generated as described under “Materials and Methods.” From this initial library, about half of the transformants (13,800) were able to rescue yeast from v-Src lethality. We next screened 2100 of these transformants for growth in the presence of 0.75 mM Inhibitor 1. At this inhibitor concentration, yeast growth was completely inhibited, and only 149 (~7%) of the 2100 transformants were found to have a significant level of growth above the controls.

Representative growth curves in the presence of Inhibitor 1 for some of these resistant colonies, as well as growth curves for the controls (yeast expressing only PTP1B, v-Src and PTP1B, and only v-Src), are shown in Fig. 2. The growth of yeast expressing only PTP1B (indistinguishable from wild-type yeast (12)) was not affected by the addition of Inhibitor 1 (Fig. 2). In contrast, no growth was observed for yeast coexpressing v-Src and a PTP1B inhibitor-resistant mutant had significant growth compared with yeast expressing PTP1B alone, yeast coexpressing only v-Src (lethal). Although they displayed a slight delay in growth compared with yeast expressing PTP1B alone, yeast coexpressing v-Src and a PTP1B inhibitor-resistant mutant had significant growth above the v-Src/PTP1B-expressing wild-type control. In fact, this delay was useful in the identification of false positives arising from the loss of v-Src expression (see clone 93 in Fig 2); ~10% of the resistant clones were false positives. When the PTP1B vector was recovered and sequenced for a number of these clones, several contained a wild-type PTP1B sequence. To confirm that the v-Src/PTP1B-expressing colonies were inhibitor-resistant, the PTP1B vector was recovered from a number of these colonies, re-transformed into v-Src-expressing yeast, and
retested for inhibitor resistance. All colonies with delayed growth were inhibitor-resistant.4

The sequences of the PTP1B mutations from 41 inhibitor-resistant clones are listed in Table 2. There were a wide variety of mutations observed, including single and multiple mutations as well as truncations. Although additional resistant colonies could be sequenced, the significant level of mutant repetition found in Table 2 suggests that this is a good representative profile of resistant mutations. For example, many inhibitor-resistant clones have Glu297, Ser295, Trp291, Ala189, Ser187, and Glu186 substitutions, suggesting that there was a high level of mutagenesis and that the screen was fairly comprehensive.

When the location and frequency of these substitutions were transposed on the PTP1B three-dimensional structure (amino acids 1–298), there was a significant clustering of mutations centering on helix α7 and including the tail of helix α6, the linker between these helices, and the top of helix α3 leading to the WPD loop and loop β9–β10 (Fig. 3). Of the 41 resistant clones sequenced, >80% contained at least one mutation within this region. In fact, alterations in helix α7 (either single point mutations or truncations due to the introduction of a stop codon or a frameshift mutation) gave rise to the majority of drug-resistant colonies.

Another very interesting observation from this screen is the absence of mutations within the active site. As mentioned above, it is fairly well understood how Inhibitor 1 interacts with PTP1B (Fig. 1). Fig. 4 shows the determinants that have been identified to affect substrate (Fig. 4A) or competitive PTP1B inhibitor (Fig. 4B) binding compared with the substitutions identified in this screen (Fig. 4C). Although there is a close correspondence between the substrate and inhibitor determinants, the mutations identified in this yeast screen completely avoided the active site. Perhaps this is not surprising given that mutations in the active site, although very likely affecting inhibitor binding, could also drastically compromise enzyme activity, resulting in yeast death. For instance, replacement of Tyr46 or Phe182 (which forms part of the hydrophobic pocket that binds the naphthalene group of Inhibitor 1) with alanine significantly reduces the $K_i$ for a DFMP inhibitor, but also significantly compromises enzyme activity (27). Even though the nearly full-length sequence of PTP1B (amino acids 1–400) was used in the screen, none of

4 J. Montalibet and B. P. Kennedy, unpublished data.
TABLE 3

Kinetic properties of the purified wild-type and S295F PTP1B enzymes

| PTP1B | pNPP | FDP | DiFMUP |
|-------|------|-----|--------|
|       | \(k_{\text{cat}}\) | \(K_m\) | \(k_{\text{cat}}/K_m\) | \(k_{\text{cat}}\) | \(K_m\) | \(k_{\text{cat}}/K_m\) | \(k_{\text{cat}}\) | \(K_m\) | \(k_{\text{cat}}/K_m\) |
| WT    | 45 ± 3 | 0.38 ± 0.02 | 12 | 2.0 ± 0.3 | 19 ± 3 | 11 | 29 ± 1 | 5.2 ± 0.2 | 5.6 |
| S295F | 18 ± 1 | 1.1 ± 0.1 | 1.6 | 0.41 ± 0.04 | 15 ± 2 | 2.7 | 34 ± 2 | 11.1 ± 0.4 | 3.1 |

The positions beyond residue 300 appeared to have much influence on inhibitor binding: no single mutation was identified within this sequence, and mutations present in this sequence all contained at least one other mutation within helix α7.

Enzyme Kinetics and Inhibitor Binding Properties of the S295F Mutant of PTP1B—Although a number of resistant clones contained multiple mutations, it is quite difficult, unless each mutation is assayed separately, to determine the contribution of each of these mutations to resistance. Because single point mutations are more easily interpreted, we decided to study in detail the enzyme kinetics and inhibitor binding properties of the S295F substitution. This position plays a central role in a strong hydrogen bond cluster linking helix α7 with the core of the enzyme (see "Discussion"); it was mutated in a couple of resistant clones in this screen and has also been identified in an earlier screen of PTP1B-(1–320) using Inhibitor 2 (see Table 4). In addition, truncation within helix α7 is enough to mediate inhibitor resistance, suggesting that such a position may be a major player in resistance.

Summarized in Table 3 are the catalytic rate constants \(k_{\text{cat}}\) and the Michaelis constants \(K_m\) for various substrates for both PTP1B-(1–320) and its S295F mutant. For the mutant enzyme, slight substrate-de-
dependent effects on \(k_{\text{cat}}\) and \(K_m\) were observed such that \(k_{\text{cat}}\) was decreased by 5-and 2.5-fold for FDP and pNPP, respectively, whereas the \(k_{\text{cat}}\) for DiFMUP was unaffected. Similarly, the \(K_m\) was slightly increased for pNPP and DiFMUP (2.9 and 1.8-fold, respectively), whereas it was unchanged for FDP. Overall, there were small reductions in the catalytic efficiency of the mutant enzyme compared with the wild-type enzyme, where the activity with the smaller pNPP substrate was more affected compared with the activities with the larger FDP and DiFMUP substrates, which were minimally affected.

Next, we evaluated the effect of this amino acid change on a panel of structurally distinct DFMP PTP1B inhibitors. Table 4 lists the inhibitor IC\(_{50}\) values for both wild-type PTP1B-(1–320) and S295F mutant PTP1B-(1–320). For all inhibitors, the IC\(_{50}\) values for the mutant enzyme were 4–5-fold higher compared with the wild-type enzyme. Further detailed inhibition studies were performed with Inhibitor 1, and it was found to be competitive with both the wild-type and mutant enzymes, with \(K_i\) values of 0.088 ± 0.003 and 0.44 ± 0.05 μM, respectively. In summary, the PTP1B S295F substitution had only a slight effect on catalytic activity, but caused a significant 5-fold shift in inhibitor affinity.

The S295F Mutation Selects against the Difluoro Moiety of DFMP PTP1B Inhibitors—How does the S295F substitution, which is located 21 Å from the active site, affect inhibitor binding to the active site? Because substrate binding to the active site was not greatly affected, it is unlikely that the active-site conformation was significantly changed. In fact, we have solved a crystal structure of the S295F mutant without inhibitor and found it to be essentially identical within the active site to the wild-type structure. Unfortunately, all attempts to crystallize this PTP1B mutant complexed with Inhibitor 1 or 2 failed. The reason for this is unclear because crystal structures for inhibitors similar to Inhibitors 1 and 2 complexed with wild-type enzyme have been obtained (see Fig. 5 and "Discussion") (24, 25).

The inhibitors used to examine the effect of the S295F mutation on inhibitor potency are structurally distinct, but all contain the same DFMP pharmacophore (Table 4). Because the IC\(_{50}\) values for all these inhibitors were similarly shifted, it is likely that this common chemical group is responsible for the loss in potency rather than the varying structural elements. Because the phosphate group is analogous to the phosphate group of the substrate and because catalysis was not significantly affected, it is unlikely that this group was responsible for the shift in potency. We decided to examine the effect of the difluoromethyl group on inhibitor potency for both the wild-type and S295F mutant PTP1B enzymes.

Table 5 lists the IC\(_{50}\) values of the wild-type and mutant PTP1B enzymes for a series of benzotriazole inhibitors that contain a difluoromethyl, monofluoromethyl, or non-fluorinated methyl phosphonate pharmacophore. With the difluoro inhibitor, there was a significant 5.5-fold difference in the IC\(_{50}\) values between the wild-type and mutant enzymes, whereas there was essentially no significant or a minimal shift for the mono and non-fluorinated inhibitors. This suggested that the S295F substitution was selecting against the difluoro moiety of these inhibitors. To further validate this, the IC\(_{50}\) values were determined for two additional PTP1B inhibitors lacking the difluoro group. One of these inhibitors, Inhibitor 8, is very similar to Inhibitor 2, except that difluoro is replaced with oxygen. Again, there was only a minimal shift in the IC\(_{50}\) values between the wild-type and mutant enzymes. An allosteric inhibitor for PTP1B has been recently described and shown to interact in the cleft between helices α6 and α3, causing a disruption in helix α7 (13). When this compound was tested, the IC\(_{50}\) values for the wild-type and mutant enzymes were basically identical. Taken together, these results indicate that the inhibitor resistance of the S295F substitution is due to a loss of affinity for the difluoro group of the DFMP inhibitors and that the kind of mutations identified in this type of screen are highly depend-

DISCUSSION

We have devised a library screen in yeast to identify regions of PTP1B that can affect the binding of DFMP inhibitors. Unlike inhibitor determinants identified by x-ray crystallography, which concentrate in and around the active site (because most PTP1B inhibitors, including the DFMP inhibitors, bind to the active site), the mutations identified in this screen were outside the active site and clustered on helices α7, α6, and α3 and loop β9–β10. The finding that this region and the S295F substitution (which is 21 Å from the active site) in particular can affect inhibitor binding is quite novel. What is also quite remarkable is the power of the selection process that can generate mutations such as S295F that can discriminate against such a subtle moiety as a fluorine atom.

The type of yeast screen was previously carried out using mammalian PDE4 and rolipram (7, 8). Two general types of determinants were obtained from the yeast PDE4 screen: those that affected only inhibitor binding and those that also altered substrate binding. At the time of these studies, no crystal structures were available, but the recent publication of the rolipram-PDE4B crystal structure (28) has provided insight.
into the mutant selection. For example, mutation of Ile$^{410}$, which is located in the active site, to threonine or asparagine results in very high rolipram IC$_{50}$ values (266- and 1000-fold increases, respectively), but also causes the largest increase in $K_m$ values (150 and 500-fold, respectively). This large decrease in rolipram potency is most likely due to the loss of the hydrophobic interaction of the phenyl group in rolipram with Ile$^{410}$ (28). In comparison, there was a complete absence of active-site substitutions in this PTP1B screen using the DFMP inhibitors (Fig. 4). This could be related to the type of inhibitor used because the DFMP inhibitors interact with all major catalytic loops of PTP1B, whereas rolipram does not completely occupy the active site and has much stronger interactions with Ile$^{410}$ compared with the substrate (28). Another possible factor is that it is not clear what level of enzyme activity is required for yeast survival. We have shown previously that the stoichiometry

TABLE 4
IC$_{50}$ values of the wild-type and S295F PTP1B enzymes using DfMUP as a substrate for a panel of five structurally different DFMP inhibitors

| Inhibitor | IC$_{50}$ Values (nM) |
|-----------|-----------------------|
|           | WT    | S295F |
| 1         | 230 ± 31 | 886 ± 380 |
| 2         | 285 ± 34 | 1644 ± 250 |
| 3         | 2.2 ± 0.3 | 11 ± 4 |
| 4         | 82 ± 13 | 399 ± 64 |
| BzN-EJJ-amide | 3.7 ± 0.5 | 18 ± 2 |

TABLE 5
Inhibitor resistance of the PTP1B S295F substitution is due to selection against the difluoro moiety of the DFMP inhibitors

| Inhibitor | IC$_{50}$ Values (nM) |
|-----------|-----------------------|
|           | WT    | S295F |
| 5         | 47 ± 7 | 260 ± 2 |
| 6         | 570 ± 130 | 830 ± 220 |
| 7         | 3260 ± 980 | 5030 ± 770 |
| 8         | 810 ± 130 | 1290 ± 240 |
| Allosteric | 9900 ± 1900 | 10900 ± 1900 |
between v-Src and PTP1B is critical such that the expression of v-Src has to be significantly attenuated compared with PTP1B to obtain good survival (12). It is possible that the PTP1B screen is more sensitive to active-site mutations than observed for the PDE screen.

The potency of DFMP as a PTP pharmacophore has been extensively analyzed (9, 15, 29, 30). Burke et al. (9) and Burke and Lee (29) have shown that the DFMP group contributes to high PTP binding affinity. Its use in the phosphonodifluoromethyl-phenylalanine peptide inhibitors demonstrated that the difluoro group can increase PTP binding by 1000-fold relative to the monofluorinated counterpart (9, 29). This is also the case for the non-peptide inhibitors, where the DFMP inhibitors are considerably more potent than their mono- and non-fluorinated counterparts (29). The fluorines also contribute to inhibitor selectivity whereby the DFMP inhibitors are highly selective for PTP1B and T-cell PTP and lose selectivity with decreased α-fluorination (15). The enhanced affinity of the DFMP inhibitors is thought to be mediated by a direct interaction with the enzyme and is not due to phosphonate pKₐ effects (15, 24).

In the crystal structure of (1,1-difluoro-1-(2-naphthalenyl)methyl)phosphonic acid complexed with PTP1B (24), the two fluorine atoms are thought to form a van der Waals contact with the phenyl ring, and the pro-R-fluorine forms a hydrogen bond with the amido group of Phe₁₈₂. Similarly, we have reported on the crystal structure of difluorobenzotriazole inhibitors complexed with PTP1B and found that the two fluorine atoms are within van der Waals distance of Phe₁₈₂ and that they are hydrogen-bonded to a water molecule that also interacts with the phosphonate, the side chain nitrogen of Gln³⁸⁸, and the main chain nitrogen of Phe₁₈₂ (25).

Examination of the crystal structures of PTP1B revealed that helix α₇ interacts with the rest of the enzyme through an extensive series of hydrogen bonds with helix α₃ and the loop between strands β₈ and β₁₀. In the S295F mutant (shown in yellow), helix α₇ is repositioned and partially disordered, with consequent disruption of this extensive network of interactions. Only the hydrogen bond between the side chains of Tyr₁₅² and Asn₁₉₃ is retained.

Along with this cluster, helix α₇ further interacts with helix α₃ by another hydrogen bond between its Gln³⁸⁸ and Glu³⁰⁰. In addition, burial of Trp⁷⁹⁷ in the hydrophobic pocket between helices α₃ and α₆ also provides stabilizing interactions. The crystal structure of the S295F mutant (shown in yellow in Fig. 5) shows that replacement of Ser²⁹⁵ with Phe results in repositioning and partial disordering of helix α₇, with consequent disruption of this extensive network of interactions. Only the hydrogen bond between the side chains of Tyr₁₅² and Asn₁₉₃ is retained, and residues such as Gln³⁸⁸ have moved as much as 6 Å from their original position. These substantial local variations probably result in a transduction of changes to Phe¹⁸₂ through helix α₃ (which is directly connected to the WPD loop; see Fig. 3) and to Gln³⁸⁶ on helix α₆, which is involved in the water-mediated binding of the inhibitors’ difluoro moiety.

Based on the structure, it is then reasonable to expect that the inhibitor resistance caused by mutations clustering on helices α₃, α₆, and α₇ is due to disruption of the interaction of helix α₇ with the rest of the enzyme core. For instance, many resistant mutations were the result of truncation of helix α₇, which would certainly interfere with this hydro- gen bond network. However, not all mutations in helix α₇ result in inhibitor resistance. For example, Gln³⁸⁶, which points outward toward the solvent and does not interact with the core residues when mutated to leucine or arginine, has no effect on catalytic activity or DFMP inhibitor binding.

Whether all mutations that cluster on helices α₃, α₆, and α₇ select against the difluoro moiety of the DFMP inhibitors remains to be explored; however, the inhibitor-resistant mutations identified in this screen are very likely DFMP- or Inhibitor 1-specific. The characterization of the S295F substitution is supportive of this and also demonstrates the feasibility of using this approach to identify novel regions of PTP1B that can influence inhibitor binding. Also, this approach is highly suited to evaluate the effect of the C terminus (amino acids 320–400) on inhibitor binding. Currently, there are no crystal structures of this region of PTP1B, and its influence on inhibitor binding has been infrequently examined (13). It was shown recently that the potency of a PTP1B allosteric inhibitor is increased with full-length PTP1B compared with the catalytic domain (13). However, for the DFMP inhibitor used in this screen, none of the amino acids beyond helix α₇ appear to have any influence on inhibitor binding; this is probably the case for all active-site inhibitors. Nevertheless, because it appears that the mutations selected in this type of screen are inhibitor-dependent, screening with additional structurally diverse inhibitors would be required to completely rule out the possibility that the PTP1B C terminus can influence active-site inhibitor binding.

In summary, we have applied a unique approach to identify regions of PTP1B that can influence DFMP inhibitor binding. Using this screen, we have shown that helix α₇ of PTP1B and Ser²⁹⁵ (not previously shown to contribute to inhibitor affinity) in particular can significantly affect the potency of DFMP inhibitors. This screen also has the additional benefit of being able to scan the nearly full-length PTP1B sequence for inhibitor determinants, and it is not restricted to PTP1B because other mammalian PTPs have been used to protect yeast from v-Src lethality and therefore could be used in this assay (16).

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