Conformation-assisted Inhibition of Protein-tyrosine Phosphatase-1B Elicits Inhibitor Selectivity over T-cell Protein-tyrosine Phosphatase Phosphatase-1B*

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PTP-1B represents an attractive target for the treatment of type 2 diabetes and obesity. Given the role that protein phosphatases play in the regulation of many biologically relevant processes, inhibitors against PTP-1B must be not only potent, but also selective. It has been extremely difficult to synthesize inhibitors that are selective over the highly homologous TCPTP. We have successfully exploited the conservative Leu to Val substitution between the two enzymes to synthesize a PTP-1B inhibitor that is an order of magnitude more selective over TCPTP. Structural analyses of PTP-1B/inhibitor complexes show a conformation-assisted inhibition mechanism as the basis for selectivity. Such an inhibitory mechanism may be applicable to other homologous enzymes.

The quest for agents that can intervene in type 2 diabetes continues to be a major research focus and challenge in many laboratories. As impaired insulin action is an underlying mechanism in type 2 diabetes, the insulin signaling pathway has naturally been the focus of research in attempts to identify suitable therapeutic target(s) for drug intervention against the disease. Insulin signaling begins with the activation of the insulin receptor (IR) via tyrosine phosphorylation and culminates in the uptake of glucose into cells by the glucose transporter, Glut4 (1). The activated IR must then be deactivated and returned to a basal state, a process that is believed to involve protein-tyrosine phosphatase-1B (PTP-1B). PTP-1B has been shown to directly interact with the activated insulin receptor (2–6). Disruption of the gene that codes for PTP-1B in mice results in sensitivity to insulin and also increased resistance to diet-induced obesity (7, 8). This phosphatase is therefore a very attractive therapeutic target for the treatment not only of type 2 diabetes but also of obesity. Predictably, there has been an intense research effort by many groups to identify potent and selective inhibitors of PTP-1B (for recent reviews, see Refs. 9 and 10). PTP-1B belongs to the protein-tyrosine phosphatase (PTP) superfamily of enzymes, which includes ~100 members involved in signal transduction and regulation of cellular processes such as growth, differentiation, and proliferation. Inhibitors against the enzyme must thus be not only potent but also selective. Sequence alignment of amino acids residues within the PTPs catalytic domain shows that, in general, all phosphatases have less than 40% identity to PTP-1B: a remarkable exception is the T-cell protein-tyrosine phosphatase (TCPTP), which has a 72% identity (11–14). Inhibitors that target PTP-1B and incorporate previously identified selectivity determinants (15–17) are generally selective (>30-fold) over all PTPs tested but are equipotent on TCPTP. Although it is not evident that co-inhibition of TCPTP (with PTP-1B) will result in serious adverse effects, mice lacking the TCPTP gene die within 3–5 weeks after birth from defects in hematopoiesis and immune function (18). Hence, it is highly desirable to design PTP-1B inhibitors that are selective over TCPTP as potential therapeutic agents particularly as type 2 diabetes is a chronic disease. The only demonstrable structural determinant(s) of inhibitor selectivity between PTP-1B and TCPTP reported to date is located in the enzyme secondary binding site (16), and inhibitors targeting that area have been shown to have some discriminatory capability between the two enzymes (19–23). Very recently, Wiesmann et al. (24) reported the discovery of a novel allosteric site that could be used for PTP-1B inhibition. Although the discovery of this site appears to be promising for the design of inhibitors with enhanced pharmacological properties, the issue of selectivity versus TCPTP remains partially addressed. The two enzymes share over 95% of sequence similarity in this area, and are structurally identical, although TCPTP lacks the phenylalanine at position 280 that appears to play a role in inhibitor binding. In order to further address the selectivity problem, we report here the identification of a previously unknown structural determinant between PTP-1B and TCPTP, and the biochemical and mutagenesis data that confirmed it. This new site may be exploited, alone or in conjunction with other selectivity determinants, to increase potency and elicit selectivity.

EXPERIMENTAL PROCEDURES

Mutagenesis, Protein Expression, and Purification—A plasmid that expresses the isolated catalytic domain of human PTP-1B, pFlag-hPTP-1B-1(1–298), was used as template DNA for the mutagenesis. The Chameleon™ double-stranded mutagenesis kit (Stratagene) was employed for all site-directed mutagenesis by following the manufacturer’s instructions. Oligonucleotides used for the site-directed mutagenesis were custom-synthesized by Invitrogen. Codons specifying the targeted amino acid changes were incorporated into the oligonucleotides as shown below (the codon changes that were introduced...
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TABLE 1
Collection and refinement statistics for the data used to solve the structures of PTP-1B WT and L119V in complex with compound 1

| Data collection statistics | WT PTP-1B | L119V |
|---------------------------|-----------|-------|
| UCP (Å)                   | 87.4, 85.6, 137.3 | 86.4, 86.6, 139.2 |
| Resolution range (Å)      | 13.0–2.2 | 21.0–2.1 |
| No. of reflections        | 53,000   | 61,477 |
| % Possible                | 99.9     | 99.6   |
| Redundancy                | 7.0      | 6.8    |
| I/σ                       | 8.8      | 12.3   |
| R_{free}/R                | 7.6      | 6.4    |

Refinement statistics

| Resolution range (Å)      | 13.0–2.2 | 15.0–2.1 |
| No. of reflections        | 49,806   | 59,205   |
| % Possible                | 94.5     | 96.3     |
| R_{free}/R                | 24.1/20.5 | 21.7/19.2 |
| R_msd bond length/angle   | 0.009/1.33 | 0.010/1.25 |
| No. protein atoms         | 2465     | 2465     |
| No. solvent atoms         | 367      | 299      |
| No. ligand atoms          | 86       | 86       |

FIGURE 1. Chemical structures and IC_{50} s for compound 1 and its analog, compound 2 (without the ester group), on PTP-1B and TCPTP.

run in each cycle. Omit maps were used to rebuild regions of the protein that were poorly defined in the initial maps. Table 1 summarizes the refinement statistics. For the structure of the mutant protein, initial σA-weighted (34) 2Fo−Fc and F_{o}-F_{c} difference electron density maps were calculated in CNX using phases derived from the WT PTP-1B:compound 1 structure, with ligand and solvent molecules removed, to check for the presence of the bound inhibitor. Refinement was then carried out essentially as described for the WT enzyme, but for the use of Refmac5 (35–38) as the refinement program. Table 1 summarizes the statistics for data collection and structure refinement.

The coordinates and structure factors for the structures described here have been deposited with the Protein Data Bank, accession codes 2FJM and 2FIN, respectively.

RESULTS

Lead Compound Identification—Our initial screening campaign of thousands of compounds did not yield any inhibitors that were selective for PTP-1B over TCPTP. Similarly, efforts to identify differences between TCPTP and PTP-1B by enzyme kinetic analysis did not reveal any discernible differences between the two enzymes that could be exploited for inhibitor design to achieve selectivity (23). Furthermore, a structural comparison of the three-dimensional crystal structures of both enzymes did not reveal obvious determinants of selectivity within...
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TABLE 2

| Kinetic parameters of PTP-1B loop mutants determined with FDP as substrate |
|-----------------------------|-----------------|-----------------|-----------------|
|                            | $k_{cat}$ | $k_{cat}/K_m$ |
| PTP-1B                     |          |                |
| Wild type                  | 20 ± 2   | 112 ± 8        | 9.3 x 10^4     |
| V113I                      | 20 ± 2   | 122 ± 17       | 1.0 x 10^5     |
| M114V                      | 22 ± 2   | 154 ± 14       | 1.2 x 10^5     |
| V113I, M114V               | 20 ± 2   | 59 ± 10        | 4.1 x 10^4     |
| G117E                      | 29 ± 7   | 96 ± 36        | 5.5 x 10^3     |
| L119V                      | 19 ± 2   | 83 ± 6         | 7.1 x 10^4     |

TABLE 3

| IC$_{50}$ values for BzN-EJJ-amide on PTP-1B wild type and loop mutants |
|-----------------|-----------------|-----------------|
| IC$_{50}$       |          |                |
| Wild type       | 8 ± 3         |                |
| V113I           | 14 ± 3        |                |
| M114V           | 13 ± 3        |                |
| V113I, M114V    | 7 ± 3         |                |
| G117E           | 11 ± 2        |                |
| L119V           | 12 ± 4        |                |

TABLE 4

| IC$_{50}$ values for compound 1 on loop mutants |
|-----------------------------------------------|
| IC$_{50}$                                    |
| Wild type                                    | 39 ± 10 |
| V113I                                        | 39 ± 8 |
| M114V                                        | 45 ± 8 |
| V113I, M114V                                 | 29 ± 3 |
| G117E                                        | 32 ± 8 |
| L119V                                        | 142 ± 41|

FIGURE 3. Comparison of the position of the 110–121 loop in the WT PTP-1B: compound 1 and PTP-1B L119V: compound 1 complexes. A, stereo-view of the loop in the WT complex: the electron density (2Fo – Fc map, contoured at 1.5 Å) is continuous and clearly indicates the presence of the loop atoms. B, stereo-view of the loop in the L119V complex: the electron density map is contoured as above. The loop as observed in the WT enzyme is shown in magenta for comparison. In this complex, the 114–122 loop is highly disordered, and electron density for residues 117–119 is not visible.

Identification of Selectivity Determinants—Using the crystal structure of PTP-1B in complex with other inhibitors as our guide (23), we hypothesized that the ester group would be likely positioned near the active site region. The structural comparison showed a high homology of 92% identity between the enzymes among the first shell residues (within 5 Å) of the inhibitor binding locus. Thus, we decided to re-assess our PTP-1B inhibitor data base to identify any compounds that showed a hint of selectivity between the two enzymes. Following a detailed and an in-depth re-evaluation of the data base, it became apparent that the presence of an ester group in a few inhibitors as exemplified by compound 1 in Fig. 1 resulted in a very modest selectivity over TCPTP. Re-evaluation of compound 1 showed a reproducible 2-fold selectivity between the enzymes. Subsequent structure-activity relationship studies revealed that the non-esterified derivative of the compound was not selective between the enzymes (compound 2, Fig. 1).

FIGURE 2. A, representation of compound 1 bound in WT PTP-1B binding site: red, P-loop or catalytic site (His214–Arg221); magenta, WPD loop (His175–Pro185), containing the catalytic Asp181; yellow, YRD loop (Tyr46–Asp48); blue, secondary ary binding site, including Arg199 and Arg234, as described by Puius et al. (16). The loop spanning residues 110–121 is colored according to atom type (C, green; N, blue; O, red, S, yellow); the residues that are different in PTP-1B and TCPTP are in orange. B, stereo-view of the overlay of compound 1 and the 110–121 loops in the two molecules present in the asymmetric unit (green and yellow). Although the conformation of the loops are somewhat different, they both assume a closed conformation (i.e. near the bound ligand), and the side chain of Leu119 is in both molecules interacting with the ligand. The position of the same loop in 1PTY (magenta) is also displayed for comparison.
sis. The recombinant proteins were purified to homogeneity and characterized kinetically. Because residues 113 and 114 are nearest neighbors and could exert a concerted effect the doubly substituted protein, V113I/M114V, was also constructed. The introduction of the amino acid substitutions did not appreciably alter the kinetic parameters of PTP-1B (Table 2). The $K_{m}$ for FDP remained unchanged for all the mutant proteins, whereas the $k_{cat}$ was within 2-fold of the value obtained for the wild-type protein. To ensure the structural integrity of the loop, we also examined the effect of the amino acid substitutions on the inhibitory potency of a nonselective compound, BzN-EJJ-amide, whose binding orientation on PTP-1B has been reported and does not involve loop 110–121 (39). No significant differences were observed between the loop mutants and wild-type PTP-1B with respect to inhibitory potency of BzN-EJJ-amide (Table 3). Thus, we conclude that the amino acid changes in loop 110–121 do not significantly alter the overall conformation of the proteins. Next, we examined the effect of the amino acid substitutions on the potency of the modestly selective compound 1. As shown in Table 4, the mutant proteins maintained affinity for compound 1 with the exception of the L119V-substituted protein. The leucine to valine amino acid substitution at position 119 resulted in a 2-fold loss in potency of compound 1, equivalent to the selectivity that was observed over TCPTP.

Crystal Structures of PTP-1B WT and L119V in Complex with Compound 1—To further understand the involvement of Leu119 in the ester-dependent selectivity and provide a structural basis for its effects, we determined the crystal structures of the isolated catalytic domains of WT-PTP-1B and of the L119V mutant in complex with compound 1. Table 1 summarizes the statistics for the refined complexes. In both cases the protein crystallized in space group P2$_1$2$_1$2$_1$, with two molecules per AU. In the WT structure, the inhibitor binds with the phosphonate in the primary binding site, or P-loop (colored in red in Fig. 2A), where the catalytic cysteine (Cys$^{215}$) is located. The benzotriazole moiety is located under the YRD loop (yellow in Fig. 2A), where it makes one hydrogen-bonding interaction with the main chain nitrogen of Asp$^{38}$. The cinnamyl moiety binds toward the secondary binding site (as defined by Puius et al., Ref. 16; blue in Fig. 2A), and the benzyl ester is located in the proximity of the loop spanning residues 110–121 (colored according to atom type in Fig. 2A). This loop, in most of the structures reported to date, was always held by interactions with symmetry-related molecules in an “open” and well ordered conformation. In the structure of BzN-EJJ-amide, the loop is not involved in interactions with other molecules, and appears to be very flexible (39). In the structures of several other inhibitors similar to compound 1 but with just a phenyl ring in proximity of the loop (23), it appears again to be disordered and/or to assume a different conformation from that observed in the 1PTY structure. However, none of these inhibitors show selectivity between PTP-1B and TCPTP as exemplified by compound 2 (see Fig. 1). In the compound 1 structure, the loop assumes two somewhat different conformations in the two molecules (Fig. 2B), but it appears in both cases to be in a “close” conformation, with the side chain of Leu$^{119}$ within van der Waals distance from the benzyl ester. The overall L119V mutant structure is extremely similar to the WT structure, and the inhibitor binds in the same fashion. The only difference is that in the mutant structure the loop 110–121 is highly disordered, and density for the residues 117–119 was not available (Fig. 3B). This further suggests that the presence of Leu$^{119}$ is necessary for ordering of the loop.

The V121L Mutant in TCPTP—Crystal structures suggested a structural mechanism for the role of Leu$^{119}$ as a selectivity determinant between PTP-1B and TCPTP. To establish whether or not that residue alone was sufficient to elicit the ester-dependent selectivity between the two enzymes, we engineered the reciprocal Val to Leu substitution into TCPTP. As shown in Table 5 (left panel), substitution of Val at position 121 to Leu resulted in a 4-fold loss in potency of compound 3, which is exemplified by compound 2 (see Fig. 1). This further suggests that the presence of Leu$^{119}$ is necessary for ordering of the loop.

**TABLE 5**

Leu to Val substitutions between PTP-1B and TC-PTP influence inhibitor selectivity

|                      | IC$_{50}$ (nM) |
|----------------------|---------------|
| Compound 1           |               |
| PTP-1B – wild type   | 39 ± 10       |
| PTP-1B – L119V       | 142 ± 41      |
| TCPTP – wild type    | 87 ± 15       |
| TCPTP – V121L        | 24 ± 2        |
| Compound 3           |               |
| PTP-1B – wild type   | 163 ± 37      |
| PTP-1B – L119V       | 1903 ± 224    |
| TCPTP – wild type    | 1600 ± 313    |
| TCPTP – V121L        | 138 ± 26      |
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121 (equivalent to position 119 in PTP-1B) elicited selectivity between the derivative and the wild-type proteins. The modest 2-fold selectivity was retained between the two derivatives as was observed for PTP-1B indicating that the residue at position 119 (PTP-1B numbering) is necessary and sufficient to confer selectivity. Also shown in Table 5 (right panel) is compound 3, a PTP-1B inhibitor that takes advantage of the loop differences and is an order of magnitude more selective over TCPTP. Absence of the oxadiazole group from this inhibitor abolishes selectivity between the two phosphatases (data not shown). In this case, the oxadiazole moiety also serves as a metabolically more stable replacement for the methyl ester of compound 1. As expected, substitution of Leu\textsuperscript{119} to Val abolishes the selectivity observed with PTP-1B over TCPTP whereas the reciprocal V121L replacement engenders a similar effect in TCPTP. Thus, the single amino acid change from Leu to Val interconverts PTP-1B and TCPTP with respect to the ester and oxadiazole-dependent selectivity.

**DISCUSSION**

We have previously reported on structural determinants on PTP-1B that mediate selectivity over other PTPs with the exception of TCPTP (15). By exploiting those determinants it was possible to synthesize inhibitors that were >30-fold selective over other PTPs and three orders of magnitude more selective over cdc25A, a dual-specificity phosphatase. Here, we have reported the identification of a selectivity determinant between PTP-1B and the structurally highly homologous TCPTP by exploiting a previously unknown characteristic of PTP-1B inhibitors, the ability to induce an ordered conformation out of the otherwise unstructured loop 110–121.

Our data suggest that the size of the inhibitor substituent near the loop influences the position of the loop itself. The side chain of Leu\textsuperscript{119} clearly interacts with the bound inhibitor, providing some degree of binding energy (compound 2, the compound 1 analog without the ester has an IC\textsubscript{50} of 109 nM, versus the 39 nM of compound 1, Fig. 1). The favorable interaction may be explained by the fact that, although the entropic factor is unfavorable during loop stabilization, there is a gain in energy obtained by removing the mostly hydrophobic loop from the solvent and locking it into a more hydrophobic environment. Substitution of Leu with Val (as in the TCPTP sequence), probably has the effect of reducing the extent of interactions between the loop and the inhibitor, with consequent loss of binding affinity, and concomitant increase of the extent of interactions between the loop and the inhibitor’s IC\textsubscript{50}. Comparison of the two structures (WT and the derivative V121L replacement engenders a similar effect in TCPTP. Thus, the single amino acid change from Leu to Val interconverts PTP-1B and TCPTP with respect to the ester and oxadiazole-dependent selectivity.

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