FEBS Letters 546 (2003) 140–148 FEBS 27378

Minireview

PTP1B: From the sidelines to the front lines!

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Received 15 April 2003; revised 19 May 2003; accepted 19 May 2003

First published online 2 June 2003

Edited by Richard Marais

Abstract Although initially viewed as housekeeping enzymes, research over the last 15 years has revealed that the protein tyrosine phosphatases (PTPs) are critical regulators of tyrosine phosphorylation-dependent signaling events and may represent novel targets for therapeutic intervention in a variety of human diseases. In this review I will describe some of the key advances in the characterization of the structure, regulation and function of the prototypic PTP, PTP1B, and illustrate how our understanding of the properties of this enzyme has revealed principles that apply to the PTP family as a whole.

Key words: PTP1B; Tyrosine phosphatase; Signal transduction; Drug development

1. Introduction

The reversible phosphorylation of tyrosyl residues in proteins is a key element of the signaling pathways induced by environmental stimuli that regulate cellular responses such as growth, proliferation, differentiation, metabolism and migration. Protein tyrosine phosphorylation is controlled through the coordinated actions of protein tyrosine kinases (PTKs) and phosphatases (PTPs). Initially it was anticipated that there would be a small number of PTPs that serve a housekeeping function; however, we have now come to appreciate that the PTPs are represented by a structurally diverse family, which in the human genome is encoded by ~100 genes, that integrates with the PTKs to maintain normal cell function [1,2]. Not unexpectedly, disturbance of the normal balance between PTK and PTP function results in aberrant tyrosine phosphorylation and has been implicated in the etiology of several human diseases, including cancer, diabetes and inflammation. The prototypic member of the PTP family is the enzyme PTP1B. This enzyme, named from a pool of PTP activity resolved by ion-exchange chromatography, was originally purified from human placenta as a 37 kDa catalytic domain [3,4]. The determination of its amino acid sequence led to the elucidation of a structural relationship with CD45 [5,6], a major transmembrane protein expressed on the surface of hematopoietic cells, and the revelation that members of the PTP family exist in receptor-like forms with the potential to regulate signal transduction through ligand-controlled dephosphorylation of tyrosyl residues in proteins. Further studies revealed that the PTP family can be subdivided into two broad categories, the classical, pTyr-specific enzymes typified by PTP1B and CD45, and the dual-specificity phosphatases, which dephosphorylate Ser/Thr as well as Tyr residues in proteins [2]. These latter enzymes, which largely maintain the same catalytic mechanism as the classical PTPs but display differences in the architecture of the active site, have been implicated in fundamentally important signaling events from control of MAP kinases in cell proliferation to the regulation of cyclin-dependent kinases in the cell cycle. These observations helped to shift the emphasis in research into the signaling function of tyrosine phosphorylation to include both PTKs and PTPs.

Since its discovery 15 years ago, continued investigation of PTP1B has revealed important insights into the structure, regulation and function of this enzyme, culminating in its recognition as a major target for a novel therapeutic strategy for the treatment of diabetes and obesity. In this review I will provide a personal perspective of some of these developments in our understanding of PTP1B and illustrate how frequently the properties of PTP1B reveal principles that apply to the PTP family as a whole.

2. The PTP1B protein: distinct catalytic and regulatory domains

Although initially purified as a catalytic domain of 37 kDa, the isolation of cDNA-encoding PTP1B revealed a full-length form of the protein that also contains an extension of 114 residues on the C-terminal side of the catalytic domain [7–9]. The C-terminal 35 residues are predominantly hydrophobic in nature and function in targeting the enzyme to the cytoplasmic face of membranes of the endoplasmic reticulum (ER) [10]. This was the first example of what has become an important concept within the PTP family, which has been referred to as the 'zip code' model [11]. The targeting of PTPs to defined subcellular locations contributes to the regulation of their function by restricting the spectrum of substrates to which they can gain access. Interestingly, alternative mRNA splicing generates additional structural, and therefore functional, diversity among several PTP gene products. For example, TC-PTP, the closest relative of PTP1B, exists in alternatively spliced forms of either 48 kDa, which, like

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Abbreviations: IGF, insulin-like growth factor; JAK, Janus kinase; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; ROS, reactive oxygen species; YB-1, Y-box binding protein-1

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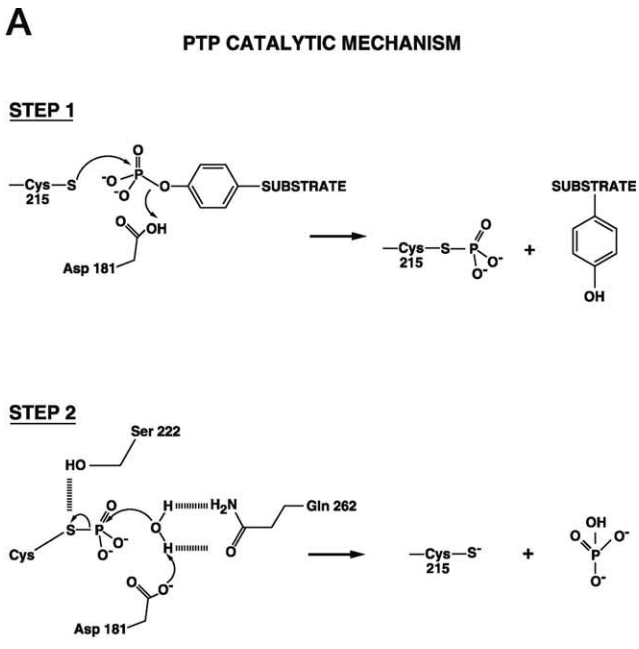
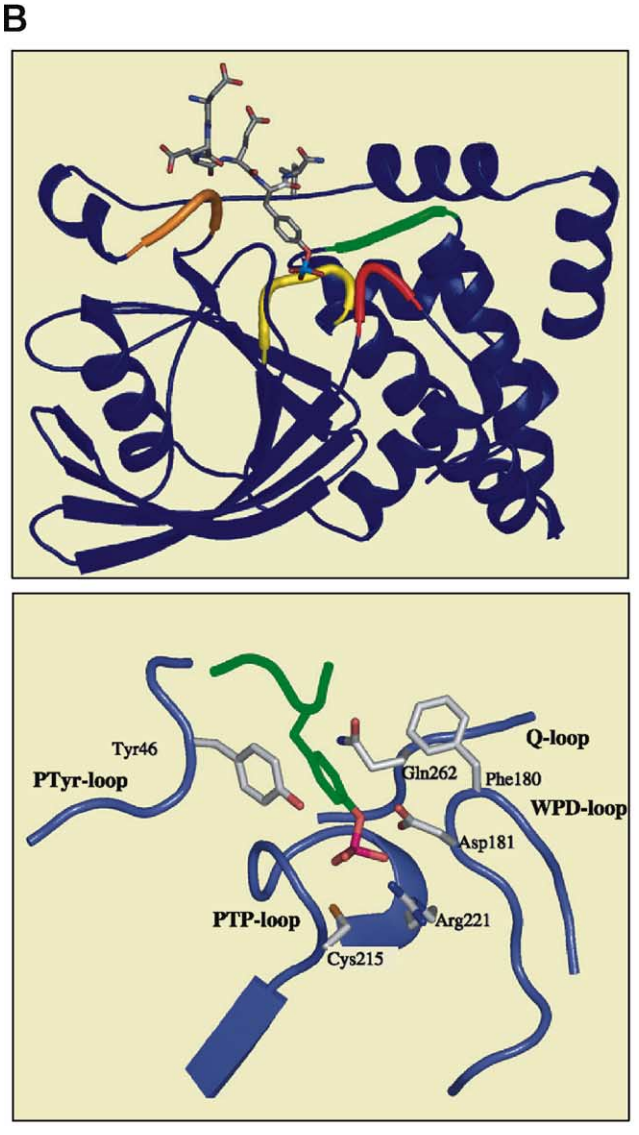
doi:10.1016/S0014-5793(03)00603-3





PTP1B, contains a hydrophobic C-terminus and is targeted to the ER, or 45 kDa, which lacks the hydrophobic segment and shuttles in and out of the nucleus under the influence of a bipartite nuclear localization signal [12,13]. Two spliced variants of PTP1B have also been reported, the relative levels of which are regulated by growth factors [14] or insulin [15]. This mRNA splicing event, which leads to retention of the last intron in the *PTP1B* gene, results in only minor differences in the sequence at the extreme C-terminus of the protein, but major differences in the 3′-UTR of the mRNA, the function of which remains to be established. It is important to realize that subcellular targeting is not the only aspect of the regulation of PTP specificity. As discussed below, the PTPs display specificity for particular substrates as a result of features intrinsic to the configuration of the PTP active site. In fact, some PTPs have been shown to display preferences for particular sites within a defined target substrate.

The C-terminal segment of PTP1B not only controls activity indirectly by regulating subcellular location, but may also exert a direct effect on catalysis. PTP1B is sensitive to proteolytic cleavage by calpain, for example following engagement of the gpIIb-IIIa integrin on the surface of platelets, which generates a truncated, soluble 42 kDa form of the enzyme [16]. Generation of this truncated form of PTP1B, which lacks ~75 residues from the C-terminal segment of the protein, including the ER-targeting motif, is associated with enhanced PTP activity, suggesting a role for the C-terminal segment in suppressing catalytic function. Such an inhibitory role has been demonstrated for the C-terminal segment of the closely related enzyme TC-PTP [17]. Whether aberrant proteolytic cleavage and activation of PTP1B occurs under pathophysiological conditions is currently under investigation. Interestingly, PTP1B was one of the first PTPs shown to exist as a phosphoprotein in vivo. Several Ser/Thr phosphorylation sites were identified in the regulatory C-terminal segment, including phosphorylation of Ser 378 by PKC (protein kinase C)and two sites, Ser 352 and 386, whose phosphorylation was regulated in a cell cycle-dependent manner [18]. Although changes in activity were associated with changes in phosphorylation, the precise mechanism by which PTP1B function may be regulated by phosphorylation is unclear. Nevertheless, phosphorylation of members of the PTP family by protein Ser/Thr kinases is now recognized as an important regulatory mechanism, with examples of both activation and inhibition of PTP function. Interestingly, this represents a mechanism of potential cross-talk between signaling pathways, whereby activation of protein Ser/Thr kinases may lead to changes in tyrosine phosphorylation through effects on PTP function. There have even been reports of phosphorylation of tyrosyl

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Fig. 1. Mechanism of action and architecture of the PTP1B active site. A: Schematic representation of the catalytic mechanism of PTP1B. B: Structure of the active site of PTP1B. The upper part of panel B illustrates a ribbon representation of the complex between PTP1B and a hexapeptide substrate, modeled on an autophosphorylation site of the EGF receptor [23]. The secondary structure elements of the PTP1B catalytic domain are shown in blue, with the critical elements that comprise the catalytic site highlighted as the signature motif (yellow), WPD loop (red), pTyr loop (orange) and Q loop (green). The lower panel illustrates a more detailed view of the active site. The pTyr substrate peptide is shown in green and the other structural elements and critical residues are labeled.

residues in PTPs, including PTP1B [19,20], although the functional significance of these modifications largely remains to be established.

3. PTP1B structure and mechanism

A variety of structural studies, coupled with extensive enzymatic and kinetic analyses from several laboratories, have provided important insights into the mechanism of substrate recognition and catalysis [21]. PTP-mediated catalysis proceeds via a two-step mechanism (Fig. 1A). All members of the PTP family are characterized by the presence of a signature motif, [I/V]HCXXGXXR[S/T], which contains the cysteinyl residue (Cys 215 in PTP1B) that is essential for catalysis. In the first step, there is nucleophilic attack by the sulfur atom of the thiolate side chain of the Cys on the substrate phosphate, coupled with protonation of the tyrosyl-leaving group of the substrate by the side chain of a conserved acidic residue (Asp 181 in PTP1B) acting as a general acid. This leads to formation of a cysteinyl-phosphate catalytic intermediate. In the second step, mediated by Gln 262, which coordinates a water molecule, and Asp 181, which functions as a general base, there is hydrolysis of the catalytic intermediate and release of phosphate.

The first crystal structure to be solved for a member of the PTP family was that of the 37 kDa PTP1B catalytic domain [22]. It was solved using sodium tungstate, an analog of phosphate and catalytic site-directed inhibitor of PTP1B, as the heavy atom derivative. The structure revealed a single domain with the polypeptide chain organized into eight α-helices and 12 β-strands with a 10-stranded mixed β-sheet that adopts a highly twisted conformation, spanning the entire length of the molecule [22]. The signature motif, which forms the phosphate recognition site, is located at the base of a cleft. The sides of the cleft are formed by three motifs (Fig. 1B). The WPD loop contains the invariant Asp residue (Asp 181 in PTP1B), which is involved in both steps of catalysis. The Q loop contains Gln 262, which mediates hydrolysis of the cysteinyl-phosphate catalytic intermediate. The pTyr loop contains a tyrosine residue (Tyr 46 in PTP1B), which defines the depth of the cleft and contributes to the absolute specificity that PTP1B displays for phosphotyrosine-containing substrates, since the smaller phosphoserine and phosphothreonine residues would not reach down to the phosphate binding site.

Further insights into substrate recognition were revealed by the structure of a complex between a peptide, representing an autophosphorylation site of the epidermal growth factor receptor, and a catalytically inactive mutant form of PTP1B, in which the essential Cys from the signature motif, Cys 215, was replaced by Ser, and which retains the ability to bind substrate even though it could not dephosphorylate it [23]. In this case, we observed that substrate binding was accompanied by a large conformational change in which the WPD loop closes around the side chain of the pTyr residue of the substrate. This causes a Phe residue (Phe 182) to stack against the phenyl side chain of the substrate pTyr residue, thus stabilizing the closed conformation of the loop, and positions the invariant Asp residue (Asp 181) to function as a general acid in the first step of catalysis. In fact, PTP1B represents an example of the concept of 'induced fit' – substrate binding induces a conformational change that creates the catalytically competent form of the enzyme.

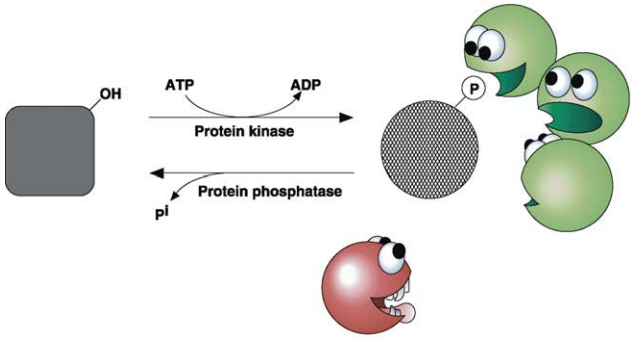

Fig. 2. Substrate-trapping mutant PTPs. Mutation of the residue that functions as a general acid in the first step of catalysis, Asp 181 in PTP1B, to Ala, creates a catalytically impaired (toothless) mutant PTP, which maintains a high affinity for substrate but does not catalyze dephosphorylation effectively. Following expression, the mutant PTP binds to its physiological substrates in the cell, protecting them from dephosphorylation by the endogenous wild-type PTP. The complex can be isolated and the substrates identified. Since the general acid is conserved in all members of the PTP family, the strategy may be applicable to all PTPs.

The structure of a PTP1B–orthovanadate complex, which is a mimic of the pentavalent phosphorus transition state, revealed H-bonding interactions between vanadate oxygen atoms and Gln 262 and Asp 181, focusing attention on these residues as playing a role in hydrolysis of the cysteinyl-phosphate catalytic intermediate [24]. We generated a Gln 262→Ala mutant form of PTP1B, which allowed trapping and visualization of the catalytic intermediate in a crystal of the mutant protein because its hydrolysis is impaired [24]. The structure confirmed the roles of Gln 262 and Asp 181 in facilitating cysteinyl-phosphate hydrolysis. It also revealed that the WPD loop is closed over the entrance to the active site, thereby sequestering the phosphocysteine intermediate with water molecules at the catalytic center and preventing the transfer of phosphate to extraneous phosphoryl acceptors. This explains why the second step in catalysis involves hydrolysis, with the PTPs being unable to phosphorylate other phosphate acceptors. In summary, through this analysis we succeeded in visualizing each of the reaction steps in PTP-mediated catalysis.

4. The generation of 'substrate trapping' mutant PTPs

The identification of substrates of PTPs is an essential step towards a complete understanding of the physiological function of members of this enzyme family. Although mutation of the active site Cys to Ser or Ala allows some PTPs to form a complex with a substrate, frequently this interaction is too weak to permit isolation of the complex, particularly from cell lysates. Using the structural insights described above, we defined several residues in the enzyme that were important for substrate recognition and catalysis and characterized the function of these residues further by site-directed mutagenesis. In so doing, we generated a form of PTP1B that maintains a high affinity for substrate but does not catalyze dephosphorylation effectively, i.e. we converted an extremely active enzyme into a 'substrate trap' [25]. Furthermore, the residue that is mutated to generate the substrate-trapping mutant is the invariant catalytic acid (Asp 181 in PTP1B) that is conserved in all members of the PTP family. Therefore, this has afforded

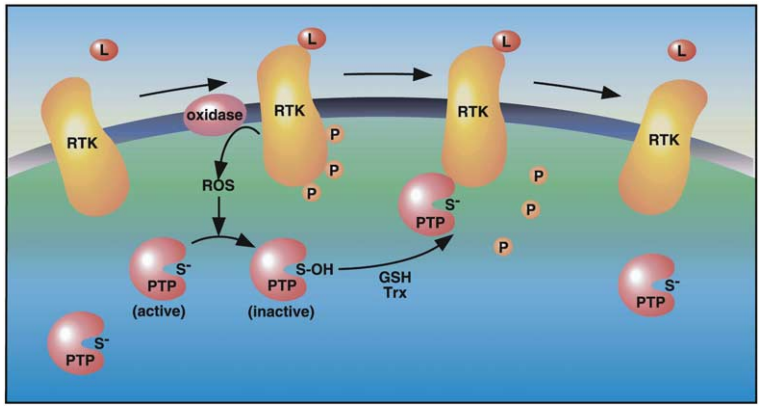

Fig. 3. Regulation of PTP activity by reversible oxidation. Ligand-dependent activation of a receptor PTK triggers a Rac-dependent NADPH oxidase leading to the production of ROS. ROS oxidize the active site Cys residue of members of the PTP family, converting it from a thiolate ion (the active form) to sulfenic acid. Oxidation results in inhibition of PTP activity, thereby promoting tyrosine phosphorylation. The sulfenic acid form of the active site Cys residue is rapdly converted to a sulfenyl-amide, protecting it from further, irreversible oxidation. This oxidation of the PTPs is transient and the enzymes are restored to their active state due to the action of glutathione or thioredoxin, leading to termination of the tyrosine phosphorylation-dependent signal. A variety of growth factors, hormones and cytokines induce ROS production and stimulate tyrosine phosphorylation. By identifying which PTPs are oxidized in response to physiological stimuli one can establish links between those PTPs and the regulation of defined signaling pathways triggered by the stimuli.

us a unique approach to identification of physiological substrates of PTPs in general (Fig. 2). Following expression, the mutant PTP binds to its physiological substrates in the cell but, because it is unable to dephosphorylate the target efficiently, the mutant and substrate become locked in a stable, 'dead-end' complex. Potential substrates can be identified by immunoblotting lysates of cells expressing the mutant PTP with antibodies to pTyr to reveal proteins whose phosphorylation state is altered as a consequence of expression of the mutant. In addition, the complex between the mutant PTP and the pTyr substrate can be isolated by immunoprecipitation and associated proteins identified by immunoblotting or, on a larger scale, by primary sequence determination. Several laboratories, including my own, have now applied this strategy to the characterization of the physiological substrate specificity of several PTPs, with intriguing results. The major take home message from this work is that members of the PTP family display exquisite substrate specificity in a cellular context.

Our original studies using the substrate-trapping mutant form of PTP1B (PTP1B-D181A) indicated a role for the enzyme in dephosphorylation of the EGF receptor PTK [25]. In that analysis we demonstrated by immunofluorescence that ectopically expressed PTP1B trapping mutant and endogenous EGF receptor colocalized in punctate structures in COS cells. More recently, an elegant study using fluorescence resonance energy transfer in PTP1B−/− fibroblasts reconstituted with PTP1B substrate-trapping mutant demonstrated that dephosphorylation of EGF and platelet-derived growth factor (PDGF) receptors occurred at specific sites in the ER, represented by these punctate structures, and required endocytosis of the PTK [26]. The localization of PTP1B to the ER frequently led to doubts being raised regarding its importance as a regulator of PTK signaling. Now these data indicate that PTP1B is present in a 'dephosphorylation compartment' that is encountered by down-regulated PTKs before being directed either to the lysosome or recycled to the plasma membrane. This suggests that PTP1B acts to terminate ligand-induced receptor-PTK signaling, rather than controlling the basal phosphorylation status of the receptor at the plasma membrane.

5. Regulation of PTP function by reversible oxidation

A substantial body of data now emphasizes the importance of production of reactive oxygen species (ROS) as a mechanism for fine-tuning tyrosine phosphorylation-dependent signaling pathways through transient oxidation and inactivation of members of the PTP family. Once again, PTP1B was a focus of early attention in this area. Due to the unique environment of the PTP active site, the essential Cys residue of the signature motif displays an unusually low p$K\alpha$ and is present predominantly as the thiolate anion at neutral pH. Although this enhances its nucleophilic properties, it also renders the PTPs susceptible to oxidation with concomitant inhibition of activity. Pervanadate inhibits PTP1B by oxidation of the active site Cys to sulfonic acid, the triply oxidized ($-SO_3H$) form [27]; however, this, and oxidation to sulfinic acid ($-SO_2H$), is an irreversible modification. In contrast, H_2O_2, a ROS produced in response to a variety of physiological stimuli, leads to oxidation of PTPs to a stable, singly oxidized sulfenic acid (–SOH) [28–30]. Again, this inhibits activity because the Cys can no longer function as a nucleophile, but this modification is reversible and therefore can form the basis for a mechanism for transient inhibition of PTP activity. Interestingly, PTP1B has now been shown to be oxidized reversibly under physiological conditions, for example in response to EGF [31] and insulin [32].

One function of ROS generated in response to a physiological stimulus is to inactivate the critical PTP(s) that provide an inhibitory constraint upon the signal transduction pathway induced by that stimulus, thereby enhancing tyrosine phosphorylation and facilitating the signaling response (Fig. 3). Our approach to this aspect of PTP regulation has been to harness ligand-induced oxidation as a mechanism for 'tagging' and thereby identifying those PTPs that are integral to the regulation of the signaling events induced by that ligand. In this regard we developed a modified 'in-gel' PTP assay to

visualize PTP oxidation in response to cell stimulation. In contrast to the effects of H_2O_2, which induced oxidation of multiple PTPs, the effects of physiological ligands are much more specific. This approach revealed an inhibitory role for SHP2 in the regulation of PDGF receptor signaling [29]. More recently, we have observed that insulin induced the reversible oxidation of both PTP1B and TC-PTP and demonstrated that, like PTP1B, TC-PTP is an inhibitor of signaling through the insulin receptor (manuscript submitted).

A fundamental issue for the role of reversible oxidation as a means of regulating PTP function is the mechanism by which oxidation produces the sulfenic acid form of the active site Cys, while irreversible inhibition by formation of higher-oxidized species is prevented. Glutathionylation of Cys 215 in PTP1B was demonstrated and suggested as such a mechanism [33]. We took a structural approach to the problem and have shown that following oxidation of PTP1B with H_2O_2, the sulfenic acid intermediate is rapidly converted to a sulfenyl-amide species [34]. A covalent bond is formed between the sulfur atom of the active site Cys and the main chain nitrogen of the adjacent residue, Ser 216, leading to formation of a novel five-atom ring structure at the active site. The consequences for the architecture of the active site are profound. The PTP loop, containing the signature motif, and Tyr 46, from the pTyr loop, flip out of the active site to adopt solvent exposed positions, but these effects are readily reversible upon incubation of the crystals with reducing agent. This novel oxidation-dependent post-translational modification, which also occurs in solution, disrupts substrate recognition by PTP1B and renders Tyr 46 susceptible to phosphorylation. Perhaps some of the reports in the literature of tyrosine phosphorylation of members of the PTP family reflect oxidation of the enzymes. Furthermore, the conformational change in PTP1B that accompanies sulfenyl-amide bond formation exposes the oxidized Cys to reducing agents to facilitate reversion to the active form of the enzyme [34]. It will be interesting to ascertain whether this represents a general mechanism for the regulation of PTP function by reversible oxidation.

6. PTP1B and oncogenesis

Due to the existence of oncoprotein PTKs, there has long been interest in the potential role of PTPs as tumor suppressors and regulators of oncogenic transformation. Expression of PTP1B in NIH3T3 cells was shown to suppress transformation by the PTKs Neu [35] and v-Src [36]. Interestingly, it has now been identified as the major PTP that dephosphorylates and activates c-Src in human breast cancer cell lines [37]. PTP1B has also been implicated in the control of cell adhesion, via effects on integrin signaling [38] and through regulation of the adhesive properties of cadherin–catenin complexes [39]. Changes in the level of expression of PTP1B have been noted in several human diseases, particularly those associated with disruption of the normal patterns of tyrosine phosphorylation, including certain cancers. My lab became interested in this area when we demonstrated that the expression of PTP1B is induced specifically by the p210 Bcr-Abl oncoprotein, a PTK that is directly responsible for the initial manifestations of chronic myelogenous leukemia [40]. These effects coincided with the ability of PTP1B to antagonize p210 Bcr-Abl-induced transformation [41]. Interestingly, expression of TC-PTP was not induced and, despite its structural similarity to PTP1B, TC-PTP did not antagonize p210 Bcr-Abl function [40,41].

The induction of PTP1B expression by p210 Bcr-Abl occurs at the level of transcription, which prompted us to characterize the *PTP1B* promoter. We found two elements that are important for expression from the human *PTP1B* promoter. We identified a *p*210 Bcr-Abl *r*esponsive *s*equence (PRS), which was required for stimulation of activity in response to the PTK [42]. The PRS is contained in a sequence that displays features of a stress response element and functions as a binding site for three mammalian C_2H_2 zinc finger proteins, Egr-1, Sp1 and Sp3. Of these, both Sp1 and Sp3 function as positive regulators, whereas Egr-1 represses Sp3-mediated transactivation of the *PTP1B* gene. Furthermore, expression of p210 Bcr-Abl results in down-regulation of the levels of Egr-1. Our data illustrate that the reciprocal actions of Sp1/Sp3 and Egr-1 are an important aspect of the regulation of PTP1B expression in response to the p210 Bcr-Abl oncoprotein [42]. In addition to the PRS, we also characterized an enhancer sequence and identified the transcription factor Y-box binding protein-1 (YB-1) as a *PTP1B* enhancer binding protein [43]. The nucleic acid binding domain of YB proteins is highly conserved throughout evolution from bacteria to humans, displaying 43% identity with the cold shock response protein cs7.4 from *Escherichia coli*, suggestive of a fundamentally important function. YB proteins bind single- and double-stranded DNA and RNA and have been implicated in both transcriptional activation and repression, functioning directly as transcription factors and indirectly by altering promoter conformation. Although originally identified in terms of their ability to recognize a DNA sequence termed a Y box, the YB proteins are now known to recognize motifs with little similarity to the classical Y box. In fact, there is no recognizable Y-box motif in the *PTP1B* enhancer and so this interaction could not have been predicted from analysis of the promoter sequence.

Using Rat1 fibroblasts, we observed that overexpression of YB-1 led to an increase in the levels of PTP1B. Furthermore, depletion of YB-1, by expression of a specific antisense construct, led to an $\sim$70% decrease in expression of PTP1B, but no change in the level of TC-PTP. Expression of antisense YB-1 resulted in increased sensitivity to insulin and enhanced signaling through the cytokine receptor gp130, which was suppressed by re-expression of PTP1B. These data illustrate that by targeting YB-1, the levels of PTP1B and signaling through insulin and cytokine receptors can be manipulated [43]. Interestingly, we observed a correlation between the expression of YB-1 and that of PTP1B across a panel of cancer cell lines. Furthermore, we demonstrated that the reported increases in the expression of PTP1B in skeletal muscle of Goto–Kakazaki non-obese, insulin-resistant type II diabetic rats, compared to control non-diabetic rats, coincided with increased expression of YB-1. These data are consistent with a functional relationship between PTP1B and YB-1 in both physiological and pathophysiological conditions and suggest that it may be possible to manipulate PTP1B activity therapeutically by controlling its level of expression [43].

7. PTP1B and the regulation of metabolic signaling

The above data touch on some of the most exciting recent developments in the study of PTP1B. Early characterization

of the enzyme purified from human placenta illustrated its ability to antagonize insulin signaling when injected into *Xenopus* oocytes [44,45]. Since then a variety of studies have explored regulatory links between PTP1B and the insulin receptor, including the use of neutralizing antibodies, overexpression and substrate-trapping strategies. Furthermore, there have been several reports of alterations in the levels of expression and activity of various PTPs, including PTP1B, in models of diabetes and obesity, as well as in clinical specimens (reviewed in [46,47]). Although the details of these reports are inconsistent, more recent analyses of quantitative trait loci and mutations in the *PTP1B* gene in humans support the concept that aberrant expression of PTP1B may contribute to diabetes and obesity. For example, a mutation in the 3′-UTR of the *PTP1B* gene, which leads to stabilization of its mRNA, has been associated with increased insulin resistance [48]. Two additional mutations in the coding region of PTP1B, one of which disrupts a potential regulatory phosphorylation site in the C-terminal segment of the protein, are also associated with type II diabetes [49,50]. Perhaps the most exciting data, however, have come from the development of *PTP1B* knockout mice.

In a landmark paper, it was shown that ablation of the *PTP1B* gene yielded mice displaying characteristics suggesting that inhibition of PTP1B function would be an effective strategy for the treatment of diabetes and obesity [51]. The PTP1B−/− mice display enhanced sensitivity to insulin. In the fed state the levels of serum glucose and insulin were lower in the knockouts than the wild-type littermates and they showed enhanced sensitivity in glucose and insulin tolerance tests. These effects coincided with enhanced tyrosine phosphorylation of the insulin receptor in muscle and liver. More surprising was the observation that ablation of PTP1B conferred resistance to obesity induced by a high-fat diet [51]. A parallel study, utilizing a different strategy to disrupt the *PTP1B* gene, targeting exon 1 rather than exons 5/6 as in [51], yielded very similar results [52]. This latter study illustrated that the enhanced insulin sensitivity that accompanies disruption of *PTP1B* was tissue-specific, occurring in skeletal muscle but not adipose tissue. Furthermore, these mice, which are characterized by a decrease in adipocyte volume, but not adipocyte number, display increases in both basal metabolic rate and total energy expenditure. Perhaps just as important as what was observed is what was not. The mice did not show a predisposition to cancer, despite the potential of PTP1B to regulate growth factor receptor PTK signaling. For example, although there is a close structural relationship between insulin and IGF-1 (insulin-like growth factor 1) receptor PTKs, and there are data to indicate enhanced phosphorylation and activity of the IGF-1 receptor in PTP1B-deficient cell lines [53], the PTP1B-deficient mice do not display phenotypes associated with aberrant IGF-1-induced signaling. It appears that although ablation of PTP1B expression does lead to hyperphosphorylation of receptor PTKs, compensatory mechanisms are induced to prevent hyperactivation of the signaling pathways triggered by these receptors [54].

By integrating crystallographic, kinetic and peptide binding studies, we defined the molecular basis of specificity in the interaction between PTP1B and activation loop of the IR and observed that the sequence E/D-pY-pY-R/K was important for optimal recognition of the insulin receptor by PTP1B [55]. Of particular importance is the presence of tandem pTyr residues; PTP1B displays 70-fold higher affinity for tandem pTyr-containing peptides derived from the insulin receptor activation loop relative to mono-pTyr derivatives. In light of this observation, we tested whether the presence of the motif could be used to predict other physiological substrates of PTP1B. Interestingly, seven PTK subtypes contain this motif in their activation loop. We focused our analysis on the JAK (Janus kinase) subfamily of PTKs and showed that JAK2 and TYK2 are physiological substrates of PTP1B [56], illustrating that PTP1B may also be an important physiological regulator of cytokine signaling. Interestingly, the satiety hormone leptin exerts its effects through a receptor in the hypothalamus that displays the hallmarks of a cytokine receptor. On the basis of our observations, we predicted that PTP1B may normally function as a negative regulator of leptin signaling in the brain and that the resistance of PTP1B-knockout mice to high-fat diet-induced obesity may arise from effects on leptin signaling [56]. Thus, aberrant down-regulation of members of the JAK subfamily of PTKs may contribute to the resistance to weight gain displayed by PTP1B-deficient mice. Interestingly, experiments in the knockout mice have illustrated such a role for PTP1B [57,58]. The definition of consensus phosphorylation sites has proven to be an important tool in signal transduction research for the identification of substrates for protein kinases. The observation that D/E-pY-pY-K/R is a consensus substrate recognition site for PTP1B suggests that this concept may also be applicable to protein phosphatases, such as PTP1B, and that consensus recognition sites may also exist for other PTPs.

8. The development of PTP1B as a therapeutic target

As current lifestyles lead to enhanced potential for obesity, with its predilection to insulin resistance, our susceptibility to diabetes and the associated cardiovascular problems that constitute ‘metabolic syndrome’ represent one of the major healthcare challenges of the 21st century. One therapeutic strategy in this area involves the search for insulin sensitizers, drugs that can ameliorate the insulin-resistance phenotype. A problem with existing insulin sensitizers, such as the thiazolidinedione PPAR-γ agonists, is their tendency to induce weight gain. The phenotype of the PTP1B knockout mouse suggests that an inhibitor of this PTP will address both obesity and insulin resistance, thereby presenting a unique therapeutic opportunity. This potential was dramatically reinforced with the demonstration that antisense oligonucleotides to PTP1B reduced the levels of the phosphatase in liver and fat, but not in muscle, in *ob*/*ob* and *db*/*db* mice, resulting in enhanced insulin sensitivity [59].

The standard approach of high-throughput screening of libraries of small molecules to identify drug candidates presents a number of problems unique to members of the PTP family. The active site of PTPs, such as PTP1B, is highly charged, as might be expected from our understanding of the catalytic mechanism and the importance of the phosphate group in substrate binding. Consequently, many of the inhibitors described to date are themselves highly charged, thus limiting bioavailability. Furthermore, although the susceptibility of the essential active site Cys residue to oxidation underlies a mechanism for fine-tuning tyrosine phosphorylation-dependent signaling, it also renders the PTPs extraordinarily sensitive to inhibition by oxidizing agents in assays in

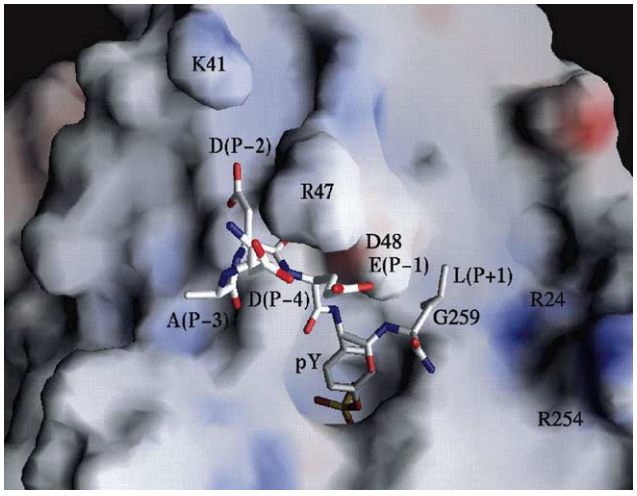

Fig. 4. Interaction of PTP1B with a synthetic peptide substrate. The figure illustrates a surface representation of PTP1B and its interaction with a hexapeptide substrate, DADEpYL, modeled on a site of autophosphorylation in the EGF receptor PTK, as described in [23]. The pTyr residue of the substrate is buried in the active site. Residues on the surface of PTP1B that have been implicated in interactions with substrates and inhibitors are highlighted.

vitro [4]. Therefore, great care must be taken in characterizing the mechanisms by which compounds inactivate these enzymes in high-throughput screening assays to avoid such non-specific oxidizing agents.

The quest for inhibitors of PTP1B has attracted the attention of a large number of laboratories from both academia and industry. Already there are 40 crystal structures of complexes of PTP1B with substrates or inhibitors in the Protein Data Bank database. These illustrate both the potential and the challenges of drug development against this target. Interestingly, structural insights into the recognition of substrates by PTP1B have revealed how specificity is achieved, raising hopes that it may also be possible to design selective inhibitors of individual PTPs. For example, PTP1B preferentially recognizes the IR activation loop that is phosphorylated on both Tyr 1162 and 1163. The pTyr 1162 residue binds to the catalytic center, while the adjacent phosphotyrosyl residue, pTyr 1163, is located within a shallow groove on the surface of PTP1B connected to the active site by a channel [55]. This was previously identified as 'a second aryl-phosphate binding site' [60], prompting much speculation about the possibility of generating inhibitors with enhanced specificity and affinity that engage both this and the catalytic site simultaneously. However, it is important to note that this 'second site' is not a clearly defined pocket like the active site. The interaction between pTyr 1163 of the substrate and PTP1B is dominated by salt bridges between the phosphate group and the side chains of Arg 24 and Arg 254 in the enzyme, with specificity for pTyr binding to this site being based upon the length of the phosphorylated residue and the positioning of these arginines [55]. Therefore, compounds that bind to this site may also be charged, thereby exacerbating the problems already experienced at the active site. In fact, attempts to design compounds that bind to both sites simultaneously have generated inhibitors with nanomolar potency, but which are highly charged and, therefore, will have limited capacity to cross the plasma membrane [61]. Other high-affinity, bidentate inhibitors have been generated that contain two pTyr mimetics; however, crystallographic analyses of their interaction with PTP1B illustrate a mode of binding that does not involve this second site [62–65]. Crystal structures of complexes of PTP1B and peptide substrates, either the IR activation loop [55] or an autophosphorylation site in the EGF receptor (Fig. 4) [23], revealed the importance of the guanidinium side chain of Arg 47 of PTP1B in hydrogen bonding to the carboxylate group of acidic residues on the N-terminal side of the pTyr substrate site. The side chain of Arg 47, a residue that is poorly conserved within the PTP family, is flexible, accommodating various combinations of residues N-terminal to the substrate pTyr site, and explaining the preference of PTP1B for acidic residues at these positions [23,66]. In fact, these bidentate inhibitors have been shown to interact with PTP1B in a similar manner to substrates, with Arg 47 playing an important role in binding the second pTyr-mimetic [62,64,65]. Interestingly, an early strategy for the design of PTP1B antagonists was to begin with peptides containing pTyr analogs, for example based on substrates, and modify them to reduce their peptidic character, creating small molecule inhibitors [67–69].

In an alternative approach, the compound 2-(oxalylamino)-benzoic acid was identified as a general, reversible and competitive inhibitor of members of the PTP family [70]. In an elegant series of studies, this compound has been modified to create specificity toward inhibition of PTP1B. In one approach, its interaction with Asp 48, a residue that is important in H-bonding interactions with main-chain N atoms in the peptide substrate [23], was manipulated. A basic nitrogen was introduced into the compound, not only to facilitate interaction between the inhibitor and Asp 48 in PTP1B, but also to induce repulsion between the inhibitor and other PTPs that contain an Asn residue in the equivalent position [71]. Additional possibilities have been explored, including exploiting the presence of a Gly residue at position 259 in PTP1B, which permits access to the second pTyr binding site adjacent to the catalytic center, in contrast to nearly all other PTPs, which have a large hydrophobic residue at the cognate position that blocks access to the site [55,72]. By derivatizing 2-(oxalylamino)-benzoic acid with a bulky group, in such a way as to engage the channel created by the presence of Gly 259, binding to PTP1B is promoted. Specificity is further enhanced by steric hindrance between the compound and the large hydrophobic residues that block the channel in the majority of the PTPs, which excludes the modified inhibitors [73]. These examples provide a dramatic illustration of the potential to generate specificity in PTP-inhibitor interactions.

9. Conclusions and perspectives

Although we have witnessed substantial progress in characterizing the structure, regulation and function of PTP1B, and in exploring strategies to inhibit that function, a drug that targets PTP1B is still a long way off. A compound termed PTP-112, which demonstrated efficacy as an anti-hyperglycemic agent in *ob/ob* mice, was withdrawn from Phase II trials due to unsatisfactory clinical efficacy and dose-limiting side effects. Similar compounds to PTP-112 activated PPAR-γ, raising the possibility of targets other than PTP1B in the cell [74]. Clearly, the development of the first PTP1B-based therapeutic will require further study.

In light of the structural similarities between the catalytic

domains of the various PTPs, and perhaps influenced by the global changes in tyrosine phosphorylation induced by the PTP inhibitor vanadate, it has been suggested that it may not be possible to generate active site-directed PTP inhibitors of sufficient specificity for therapeutic development. Interestingly, similar criticisms were leveled at the protein kinases; however, not only have we witnessed the development of protein kinase inhibitors as drugs, but also they are targeted to the ATP binding site, a feature shared by all of the kinases [75]. There are many opportunities to explore further the possibilities for building specificity into PTP inhibitors. As illustrated already in examples in the literature, there are compounds that induce closure of the catalytic site WPD loop, whereas others engage the enzyme in such a way as to maintain the open, inactive conformation of the WPD loop, with its greater binding surface. Progress to date has already highlighted the potential in further exploration of the region surrounding Arg 47 and Asp 48, as well as Gly 259 and access to the second pTyr binding site, in generating inhibitor specificity toward PTP1B [71,73]. All studies to date have focused on truncated PTP1B constructs comprising the catalytic domain. It will be interesting also to pursue the role of the regulatory C-terminal segment of PTP1B, to test whether it interacts with the active site. As this segment is less conserved than the catalytic domain, it may offer additional opportunities to exploit specificity in inhibitor design. The problem represented by the tendency for the most potent inhibitors also to be highly charged will have to be addressed. Novel pTyr mimetics [76], as well as prodrug strategies involving modification of the charged groups with neutral hydrolyzable moieties such as esters, are currently under investigation. Ultimately, it may not be possible to generate small molecule inhibitors with the appropriate combination of affinity, specificity and bioavailability, in which case powerful new technologies, such as RNA interference, may offer alternative strategies for inhibition of PTP function in a therapeutic context.

Various approaches, from the development of substrate-trapping mutants to gene ablation, have highlighted the potential for PTPs to display exquisite substrate specificity in vivo and to function as regulators of fundamentally important signal transduction events. Therefore, it is important to remember that there is more to the PTP family than just PTP1B! With the completion of the human genome sequence it has been possible to define the number of PTP genes as ~100 (see the website http://ptp.cshl.edu, and the parallel site http://science.novonordisk.com/ptp, for a detailed analysis of the PTP family). The diversity of the 'PTP-ome' is further enhanced through such mechanisms as alternative splicing, alternative promoter usage and various post-translational modifications. Additional links between PTPs and human diseases, including cancer, inflammation and infectious disease, have already been established and it is likely that more will be defined. Hopefully, further characterization will establish new functions for these enzymes and perhaps portray the PTPs as a platform for the development of novel therapeutics in a variety of disease states.

Acknowledgements: Work in my laboratory is supported by Grants R37-CA53840 and R01-GM55989 from the National Institutes of Health. I am grateful to all past and present colleagues who have contributed to the research efforts of the laboratory. I thank Jannik Andersen and Bob Del Vecchio for critical reading of the manuscript and for their help in preparing the figures.

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