



Destabilization of the SHP2 and SHP1 protein tyrosine phosphatase domains by a non-conserved “backdoor” cysteine

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ABSTRACT

Protein tyrosine phosphatases (PTPs) are critical regulators of cellular signal transduction that catalyze the hydrolytic dephosphorylation of phosphotyrosine in substrate proteins. Among several conserved features in classical PTP domains are an active-site cysteine residue that is necessary for catalysis and a “backdoor” cysteine residue that can serve to protect the active-site cysteine from irreversible oxidation. Curiously, two biologically important phosphatases, Src homology domain-containing PTPs 2 and 1 (SHP2 and SHP1), each contain an additional backdoor cysteine residue at a position of the PTP domain that is occupied by proline in almost all other classical PTPs (position 333 in human SHP2 numbering). Here we show that the presence of cysteine 333 significantly destabilizes the fold of the PTP domains in the SHPs. We find that replacement of cysteine 333 with proline confers increased thermal stability on the SHP2 and SHP1 PTP domains, as measured by temperature-dependent activity assays and differential scanning fluorimetry. Conversely, we show that substantial destabilization of the PTP-domain fold is conferred by introduction of a non-natural cysteine residue in a non-SHP PTP that contains proline at the 333 position. It has previously been suggested that the extra backdoor cysteine of the SHP PTPs may work in tandem with the conserved backdoor cysteine to provide protection from irreversible oxidative enzyme inactivation. If so, our current results suggest that, during the course of mammalian evolution, the SHP proteins have developed extra protection from oxidation at the cost of the thermal instability that is conferred by the presence of their PTP domains' second backdoor cysteine.

1. Introduction

The protein tyrosine phosphatases (PTPs), which dephosphorylate phosphotyrosine residues in protein substrates, constitute a large family of enzymes that function in mammalian cell signaling. The human genome encodes 37 classical PTPs, and misregulated classical PTP activity has been implicated in dozens of human diseases, including cancers, type 2 diabetes, hypertension, and inflammatory bowel disease [1–4].

Src homology domain-containing PTP 2 (SHP2, encoded by *PTPN11*) is a widely expressed classical PTP that has emerged as an important anti-cancer therapeutic target [5,6]. Somatic SHP2 mutations are the most common cause of sporadic juvenile myelomonocytic leukemia (JMML) and have also been observed at lower incidence in a variety of other leukemias [7] and solid tumors [8]. Moreover, germline SHP2 mutations cause Noonan and LEOPARD syndromes, both of which can lead to cancer predisposition [9–11].

SH2-domain-containing PTP 1 (SHP1, encoded by *PTPN6*) is a close

homologue of SHP2 (59% identity) that is predominantly expressed in hematopoietic and epithelial cells [12,13]. SHP1 is an important control element in B- and T-cell receptor signaling, as well as epithelial differentiation and proliferation [12,14]. Although few direct connections between SHP1 mutations and human disease have been established [15–18], targeted SHP1 inhibition has been proposed as a possible means for increasing the efficacy of anti-cancer immunotherapy [19].

Interestingly, the two SHP PTPs possess a cysteine residue at position 333 (C333) that is only found in these enzymes [1]. (333 is the amino-acid number in human SHP2. The corresponding residue in SHP1 is C327. Because SHP2 is the more widely studied PTP, this report will use SHP2 numbering when referring to amino-acid positions within the conserved PTP catalytic domain.) Among other human classical PTPs, 34 have proline at position 333 and one has serine (Fig. 1A) [1]. C333 is one of two “backdoor” cysteines in the SHPs [20]; the other (C367) is highly conserved among PTPs (Fig. 1A) [1]. Within the SHP PTP domains, C333 and C367 are located behind the active site, close in space to a conserved active-site cysteine that plays a key role in the PTP

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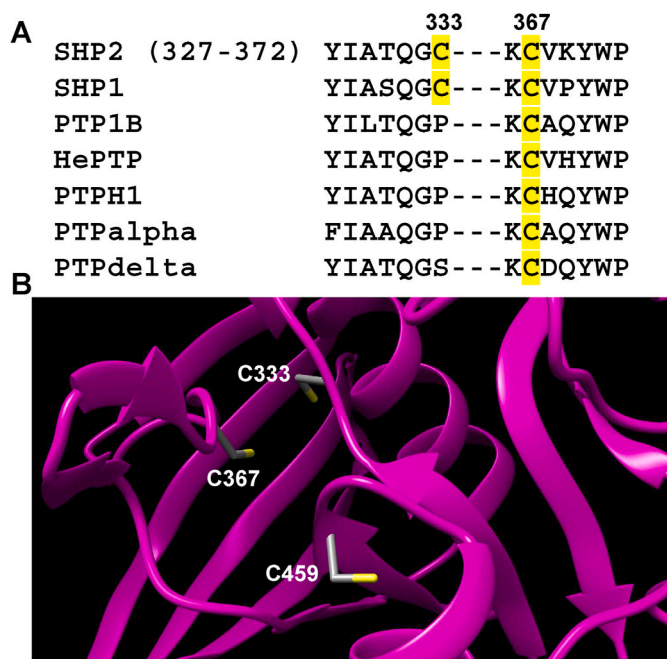


Fig. 1. The SHP PTPs contain a second, non-conserved backdoor cysteine residue. A) Partial primary-sequence alignment of SHP2, SHP1, and selected non-SHP classical PTP domains. Backdoor cysteines are highlighted. Residue numbering is according to human SHP2. The residue corresponding to SHP2's C333 in human PTP1B is P87. B) Three-dimensional structure of the SHP2 catalytic domain (PDB ID: 3B7O) [21]. The sidechains of SHP2's conserved active-site cysteine (C459), conserved backdoor cysteine (C367), and non-conserved backdoor cysteine (C333) are shown and colored by element.

catalytic mechanism and is indispensable for activity (C459 in SHP2, Fig. 1B) [21]. It has been established in multiple PTP subfamilies that the conserved C367 can serve to protect the active-site cysteine from irreversible oxidation, thereby preventing irreversible PTP inactivation under oxidizing conditions [20,22–27]. The role of the non-conserved C333 in SHP redox chemistry is less well understood [24], but it has previously been suggested that the “extra” cysteine in the SHP PTPs also participates in protection from irreversible PTP oxidation through reversible disulfide formation with C367 [20]. Since the vast majority of classical PTPs only have one backdoor cysteine, this hypothesis might suggest that the SHPs possess an additional layer of oxidative protection, as compared to other homologous PTPs.

In previous work, we have shown that the extra backdoor cysteine of the SHPs presents an opportunity for selective drug discovery, as SHP2's C333 constitutes part of an allosteric site that can be targeted with cysteine-reactive small molecules [28,29]. Here we show that the same residue imparts instability to the folded PTP domain. We find that wild-type SHPs, which contain the extra cysteine, are substantially more thermolabile than mutant SHPs in which the cysteine has been replaced with proline. Additionally, we show that instability is conferred by placement of an extra backdoor cysteine into PTP1B, a standard (non-SHP) PTP that has proline at position 333. Together, our results show that the PTP domains are substantially destabilized by the loss of proline at position 333 and suggest that the SHPs may have “selected” the oxidative-protective function of the second backdoor cysteine at the cost of protein-fold instability during the course of evolution.

2. Material and methods

2.1. General

All assays were performed in triplicate; error bars and “±” values represent the standard deviations of at least three independent

measurements.

2.2. Cloning and mutagenesis of PTP-encoding genes

The vectors encoding His₆-tagged human SHP2 catalytic domain (SHP2cd; UniProtKB Q06124, aa 224–539), His₆-tagged human SHP1 catalytic domain (SHP1cd; UniProtKB P29350, aa 243–541), His₆-tagged PTP1B catalytic domain (PTP1Bcd; UniProtKB P18031, aa 1–321), and multi-domain His₆-tagged human SHP2 (SHP2md; UniProtKB Q06124, aa 1–541), have been described previously [28–30]. Site-directed mutations were introduced using the Quikchange mutagenesis kit (Stratagene) according to the manufacturer's instructions. Desired mutations were confirmed by DNA sequencing.

2.3. Protein expression and purification

All proteins were expressed and purified using HisPur Ni-NTA resin (Thermo Scientific) per the manufacturer's instructions as previously described [28]. After purification, proteins were exchanged into storage buffer (50 mM 3,3-dimethylglutarate at pH 7.0, 1 mM EDTA, 1 mM TCEP), concentrated, flash-frozen in liquid nitrogen, and stored at –80 °C. Bradford protein assays and a NanoDrop spectrophotometer were used to measure enzyme concentrations, and SDS-PAGE was used to assess purity of protein stocks.

2.4. Phosphatase activity using para-nitrophenylphosphate (pNPP)

PTP assays using pNPP as substrate were carried out in a total volume of 200 μ L, containing PTP buffer (50 mM 3,3-dimethylglutarate at pH 7.0, 1 mM EDTA, 50 mM NaCl), enzyme (varying concentrations: see figure legends), and pNPP (varying concentrations: see figure legends) at 22 °C. PTP reactions were quenched by the addition of 40 μ L of 5 M NaOH, and the absorbances (405 nm) of 200 μ L of the resulting solutions were measured. When relevant, kinetic constants were determined by fitting the data to the Michaelis-Menten equation using SigmaPlot 12.3. For assays at elevated temperatures, all reaction components were pre-incubated on a heat block for 30 min at the test temperature before the PTP reactions were started by the addition of pNPP. Assays that tested the effect of the small-molecule aggregator miconazole contained 2.5% DMSO (vehicle). A summary of kinetic parameters for all proteins investigated is given in [Supplementary Table S1](#).

2.5. Differential scanning fluorimetry

Differential scanning fluorimetry (DSF) experiments on PTP catalytic domains were carried out in 20 mM HEPES pH 7.5, 50 mM NaCl, 1 mM TCEP, 10 μ M protein, SYPRO Orange Protein Gel Stain (purchased as a 5,000x concentrated solution in DMSO from Thermo Fisher Scientific and used at 25x), and 1% DMSO. (Multi-domain SHP2 was assayed at 25 μ M instead of 10 μ M) Measurements were carried out on 25 μ L of each solution in 96-well qPCR plates. Solutions were placed in a qPCR thermocycler and the temperature was raised by 0.5 °C every 30 s from 25 °C to 95 °C with continuous measurement of fluorescence (excitation: 472 nm; emission: 570 nm). Melt temperatures (T_m) were defined as the inflection point of the sigmoidal increase in SYPRO™ Orange fluorescence upon protein unfolding. Standard free energy of unfolding values at 298 K (ΔG_u°) were estimated from DSF data as described previously [31]. A summary of ΔG_u° values for all proteins investigated is given in [Supplementary Table S1](#). The linear fits of free energy of unfolding (ΔG_u) versus temperature that were used for extrapolation of ΔG_u° values at 298 K are shown in [Supplementary Figure S1](#).

3. Results

3.1. Instability of folded SHP2 conferred by C333

Having shown previously that C333 confers novel inhibitory sensitivity on the SHP PTPs [28,29], we sought to determine whether the presence of cysteine at position 333 affects the stability of folded PTP domains. To do so, we expressed and purified both the wild-type SHP2 catalytic domain (SHP2cd) and a mutant of the protein in which C333 was replaced by proline (C333P SHP2cd). Consistent with previous results [28], we found that the mutation had little effect on SHP2cd's intrinsic activity at ambient temperature when assayed with the small molecule PTP substrate pNPP (Fig. 2A). To investigate whether the thermal stability of SHP2cd differs from that of C333P SHP2cd, we measured the enzymes' activities over a range of temperatures at a fixed substrate concentration (Fig. 2B). We hypothesized that reaction rates of the PTP-catalyzed reactions would increase with temperature until the enzymes started to denature, at which point the reaction rates would drop off dramatically. Indeed, we observed increased reaction rates at moderately elevated temperatures, and at 37 °C and 43 °C wild-type and C333P SHP2cd displayed essentially indistinguishable catalytic activities. By contrast, the wild-type and mutant PTPs had very different catalytic properties at high temperatures. At 49 °C, SHP2cd's activity decreased relative to that of C333P SHP2cd, presumably due to partial unfolding of the wild-type protein. At 55 °C, the activity of C333P SHP2cd dropped off only slightly (compared to its high point at 49 °C), whereas wild-type SHP2cd activity decreased dramatically (Fig. 2B).

To further probe whether the temperature-dependent loss of activity in wild-type SHP2cd was due to unfolding of the polypeptide, we turned to differential scanning fluorimetry (DSF), a technique that has previously been used to measure both the unfolding temperatures (T_m) and standard free energy of unfolding values (ΔG_u°) for folded proteins [31–33]. In strong agreement with the temperature-dependent activity data, we found that wild-type SHP2cd unfolded at a substantially lower temperature ($T_m = 49.2$ °C) than did C333P SHP2cd ($T_m = 54.7$ °C, Fig. 2C). We also used the DSF data to estimate ΔG_u° values for both proteins [31] and confirmed that the wild-type protein is less stably folded by approximately 5 kJ/mol (Fig. 2C).

In contrast to the isolated SHP2 catalytic domain (SHP2cd), cellular full-length SHP2 is a multi-domain enzyme that contains two Src-homology 2 (SH2) domains in addition to its catalytic PTP domain. The SH2 domains serve to regulate SHP2's PTP activity through an autoinhibitory interaction with the enzyme's catalytic domain [34]. To control for the possibility that the increased thermal stability of C333P SHP2cd is an artifact of expressing the catalytic domain independent of the protein's regulatory domains, we expressed a multi-domain SHP2 construct that contains SHP2's SH2 domains in addition to its catalytic domain (SHP2md, comprising amino acids 1–541). (For the purposes of efficient expression from *E. coli*, SHP2md lacks the unstructured C-terminal portion of the protein, residues 542–593 [35].) We purified both wild-type and C333P SHP2md and investigated the proteins' thermal stabilities by DSF. We found that the presence of the SH2 domains increased the thermal stability of SHP2, as SHP2md displayed a T_m of 54.1 (Fig. 3), approximately 5 °C higher than that of SHP2cd (Fig. 2C). More to the point, replacement of C333 with proline in the context of the multi-domain protein gave rise to substantial increases in both T_m and ΔG_u° (Fig. 3). These data suggest that the stabilization conferred by proline in the SHP2 catalytic domain is not an artifact of protein truncation, but is intrinsic to the SHP2 structure, even within the context of the multi-domain protein.

3.2. Instability of the folded SHP1 catalytic domain conferred by C333

To investigate whether the destabilization conferred by SHP2's C333 is common to both SHP proteins, we generated a mutant of the SHP1 catalytic domain in which the extra backdoor cysteine is mutated to

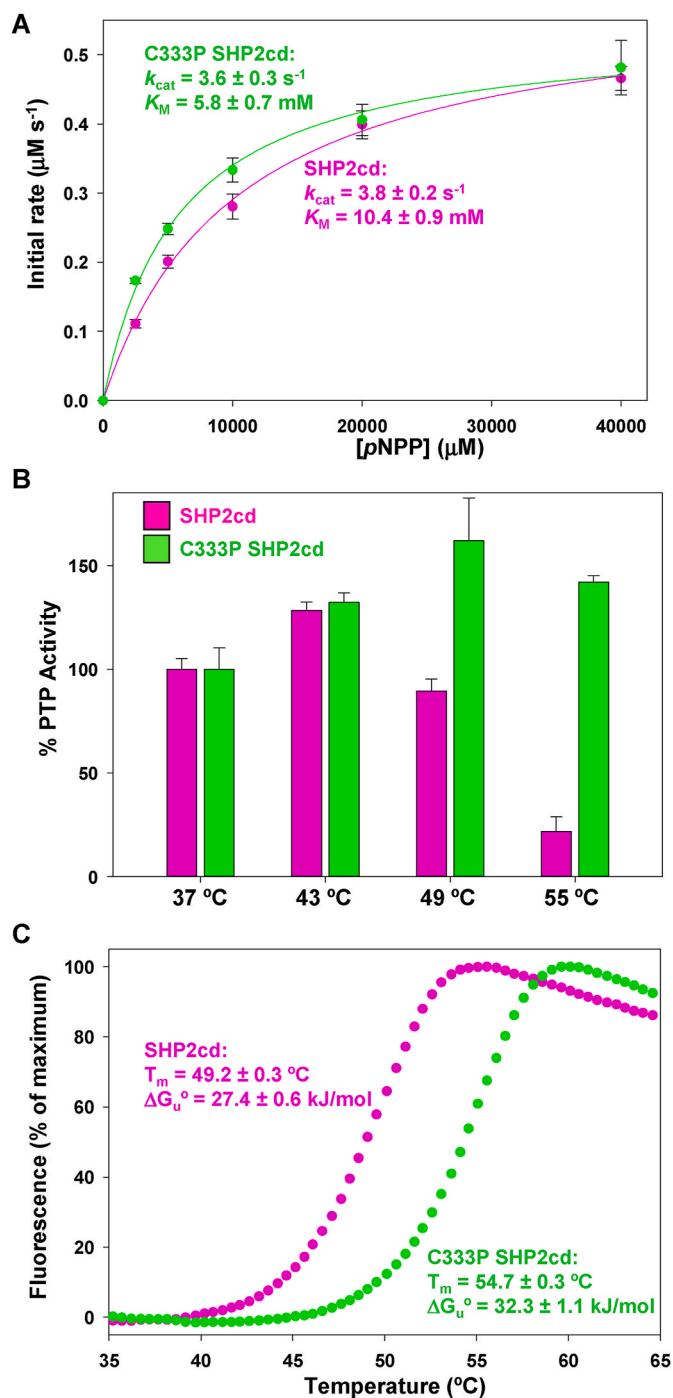


Fig. 2. A non-conserved backdoor cysteine destabilizes the fold of SHP2's catalytic domain. **A**) Michaelis-Menten kinetics at ambient temperature: SHP2cd (150 nM, magenta circles) and C333P SHP2cd (150 nM, green circles) were assayed for PTP activity at 22 °C with pNPP at the indicated concentrations. **B**) Temperature-dependent activity assay: After incubation at the indicated temperatures for 30 min, wild-type SHP2cd (250 nM, magenta bars) and C333P SHP2cd (250 nM, green bars) were assayed for PTP activity with the substrate pNPP (5 mM). Data at elevated temperatures are normalized to the activity of the corresponding enzyme at 37 °C. **C**) DSF: Wild-type SHP2cd (10 μM , magenta circles) and C333P SHP2cd (10 μM , green circles) were heated in the presence of SYPRO Orange with continuous monitoring of fluorescence (excitation: 472 nm; emission: 570 nm). Fluorescence data from one representative experiment are shown for each protein. The indicated T_m and ΔG_u° averages and standard deviations derive from three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

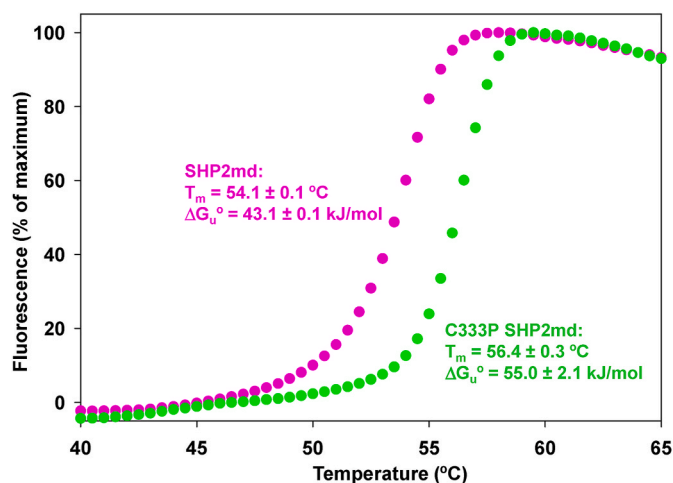


Fig. 3. C333 destabilizes SHP2's protein fold in the context of the multi-domain protein. DSF: Wild-type SHP2md (25 μ M, magenta circles) and C333P SHP2md (25 μ M, green circles) were heated in the presence of SYPRO Orange. Data were collected and analyzed as described in Fig. 2C. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

proline (C327P SHP1cd). As previously observed for SHP2cd, we found that wild-type and mutant SHP1cd had very similar kinetic properties at room temperature (Fig. 4A), but very different thermostabilities in a temperature-dependent activity assay (Fig. 4B). Whereas both enzymes were kinetically competent at 37 $^{\circ}$ C, the activity of wild-type SHP1cd dropped off dramatically at 43 $^{\circ}$ C, a temperature at which C327P SHP1cd retained its full activity. At 49 $^{\circ}$ C both enzymes lost essentially all activity, suggesting that the SHP1 catalytic domain is less thermostable than the SHP2cd, regardless of the nature of the residue at position 333.

We once again sought to corroborate the findings of the temperature-dependent activity assays by DSF. We found that the fold of C327P SHP1cd is indeed considerably more stable than that of wild-type SHP1cd, as shown by substantially increased values of both T_m and ΔG_u° in the mutant (Fig. 4C). These data show that the instability conferred by the presence of cysteine in the 333 position is not idiosyncratic to SHP2; rather, it is common to both members of the SHP subfamily of PTPs.

3.3. Destabilization of the PTP1B catalytic domain by introduction of a second backdoor cysteine

We next asked whether introduction of a non-natural cysteine could confer instability on a PTP domain that contains proline at position 333. To do so, we selected PTP1B [36,37] as a representative PTP that contains only one backdoor cysteine (Fig. 1A), and we introduced a second backdoor cysteine in the PTP1B catalytic domain to generate P87C PTP1Bcd. (The amino-acid number of "position 333" in PTP1B is much lower than in the SHPs because PTP1B does not contain regulatory domains N-terminal to its PTP domain.) We found that, compared to wild-type PTP1Bcd, the P87C mutant displayed modestly reduced catalytic efficiency at ambient temperature (Fig. 5A). Moreover, in strong agreement with our results on the SHPs, we observed that insertion of a second backdoor cysteine significantly destabilized the PTP1B catalytic domain, as demonstrated by both temperature-dependent activity assays and DSF. In activity assays, wild-type PTP1Bcd was the more stable enzyme, losing no activity at 42 $^{\circ}$ C and little activity at 47 $^{\circ}$ C, whereas P87C PTP1B activity was significantly more thermolabile at both temperatures (Fig. 5B). Corroborating the activity assays, we found that P87C PTP1B unfolded at a temperature approximately 3.5 $^{\circ}$ C lower than the wild-type protein, which also had a higher ΔG_u° value than the

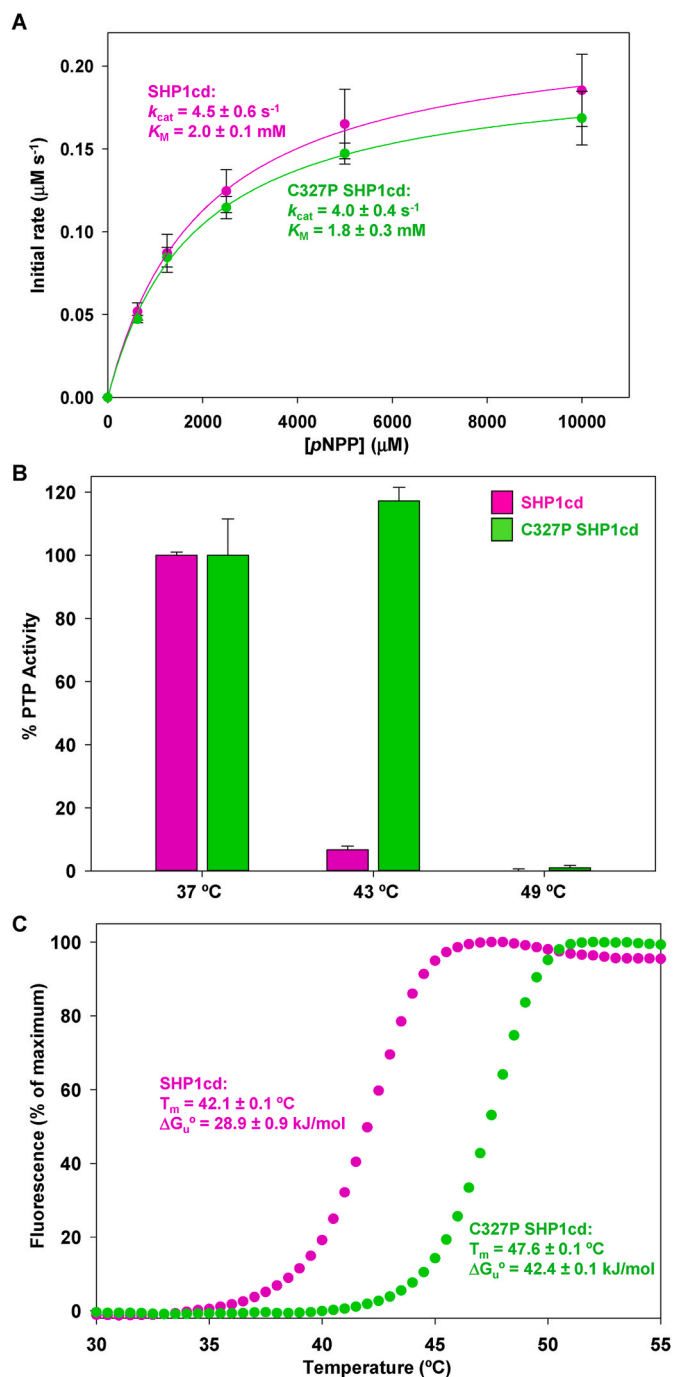


Fig. 4. A non-conserved backdoor cysteine destabilizes the fold of SHP1's catalytic domain. A) Michaelis-Menten kinetics at ambient temperature: SHP1cd (50 nM, magenta circles) and C327P SHP1cd (50 nM, green circles) were assayed for PTP activity at 22 $^{\circ}$ C with pNPP at the indicated concentrations. B) Temperature-dependent activity assay: After incubation at the indicated temperatures for 30 min, wild-type SHP1cd (250 nM, magenta bars) and C327P SHP1cd (250 nM, green bars) were assayed for PTP activity with the substrate pNPP (5 mM). Data at elevated temperatures are normalized to the activity of the corresponding enzyme at 37 $^{\circ}$ C. C) DSF: Wild-type SHP1cd (10 μ M, magenta circles) and C327P SHP1cd (10 μ M, green circles) were heated in the presence of SYPRO Orange. Data were collected and analyzed as described in Fig. 2C. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

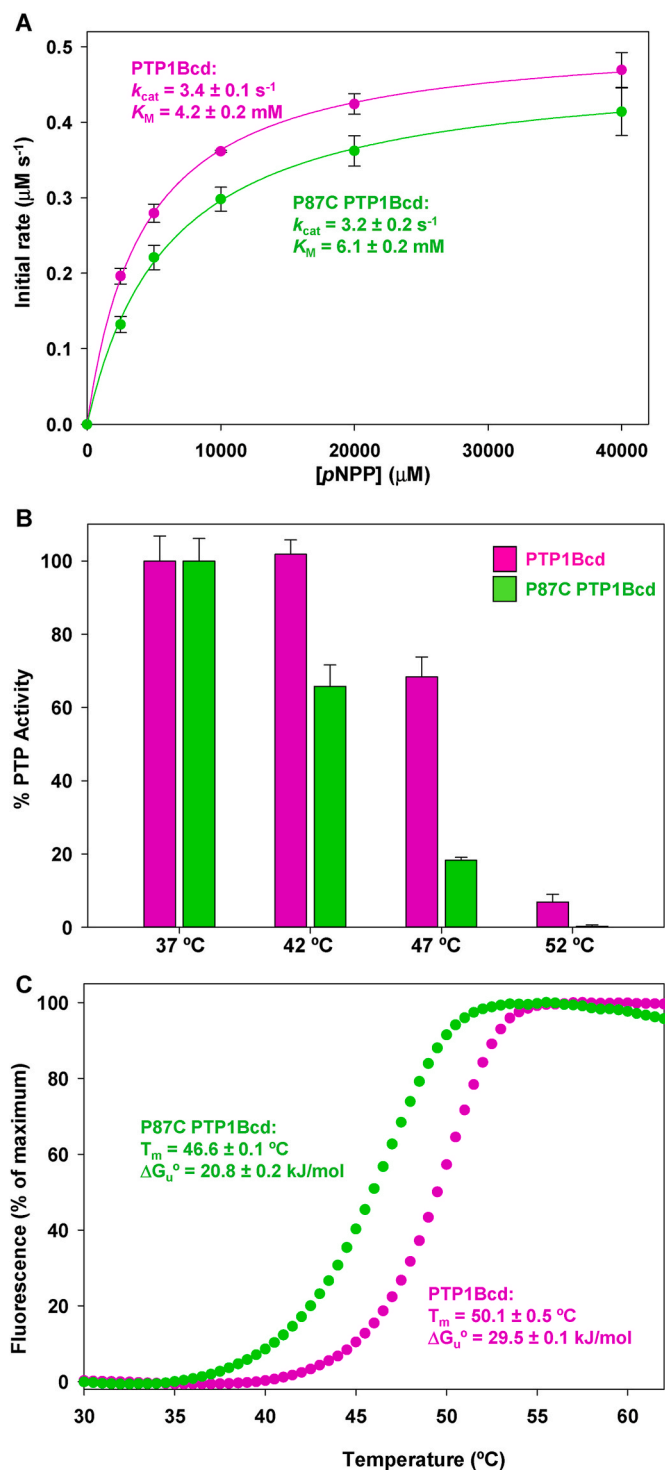


Fig. 5. The PTP1B catalytic domain is destabilized by introduction of a second backdoor cysteine. **A**) Michaelis-Menten kinetics at ambient temperature: PTP1Bcd (150 nM, magenta circles) and P87C PTP1Bcd (150 nM, green circles) were assayed for PTP activity at 22 °C with pNPP at the indicated concentrations. **B**) Temperature-dependent activity assay: After incubation at the indicated temperatures for 30 min, wild-type PTP1Bcd (250 nM, magenta bars) and P87C PTP1Bcd (250 nM, green bars) were assayed for PTP activity with the substrate pNPP (5 mM). Data at elevated temperatures are normalized to the activity of the corresponding enzyme at 37 °C. **C**) DSF: Wild-type PTP1Bcd (10 μM , magenta circles) and P87C PTP1Bcd (10 μM , green circles) were heated in the presence of SYPRO Orange. Data were collected and analyzed as described in Fig. 2C. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

mutant (Fig. 5C). These data show that proline at position 333, which is present in the majority of classical PTPs, serves to stabilize the PTP-domain's fold. The destabilization conferred by replacement of proline with cysteine in three PTP domains (SHP2, SHP1, and PTP1B), with no exceptions, suggests that proline 333 also acts as a common stabilizing feature across other members of the classical PTP family.

4. Discussion

In the current study, we have investigated the effects of the SHP-specific backdoor cysteine on protein-fold stability within the conserved classical PTP domain. We find that the extra cysteine at position 333 confers thermal instability (“C333 instability”) in the context of both SHP2 and SHP1. Viewed from the opposite perspective, our data show that the conserved proline at position 333 serves to stabilize the catalytic-domain fold in non-SHP PTPs.

What are the ramifications of C333 instability in the SHPs? Because the SHPs are intriguing targets for therapeutic intervention—SHP2 is arguably the most important drug target among all PTPs [38]—we wondered if C333 instability might affect the enzymes' responses to small molecules. Specifically, it has previously been shown that protein instability correlates with sensitivity to non-specific small-molecule aggregators [39]. We hypothesized that C333 instability might render the SHPs particularly prone to aggregation, potentially complicating the search for selective SHP inhibitors. As a first test of this idea, we assayed the sensitivity of both wild-type and C333P SHP2cd to the non-specific aggregator miconazole [40]. We detected only a marginal difference in miconazole sensitivity between the thermolabile SHP2cd and thermostable C333P SHP2cd (Fig. 6), suggesting that C333 instability does not dramatically affect SHP2's sensitivity to non-specific aggregation. Further studies would be required to more fully assess the relationship between thermal stability and aggregator sensitivity in the SHPs and other PTPs.

Why is the destabilizing C333 present in the SHPs? While our data clearly establish the role of proline 333 in stabilizing non-SHP PTP domains, the function of the destabilizing C333 in SHP biochemistry remains less apparent. Chen and co-workers have presented evidence that C333 plays a role in preventing irreversible oxidation of the SHPs' catalytic cysteine (C459) [20]. Specifically, these authors detected the formation of C333–C367 disulfide bonds in SHP catalytic domains that

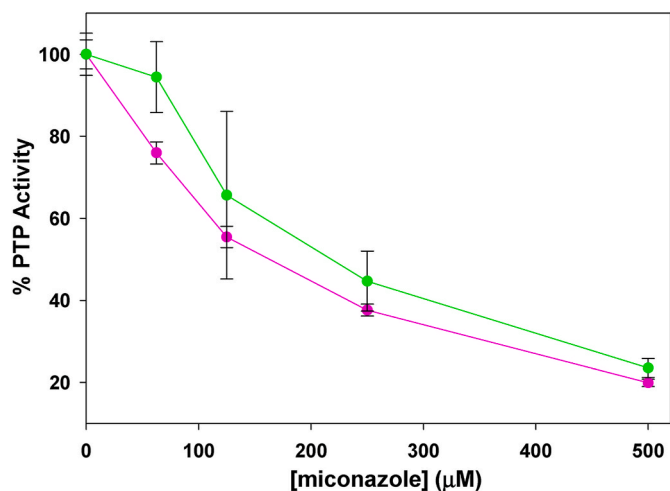


Fig. 6. Dose dependence of SHP2 catalytic-domain inhibition by miconazole. After a 15-min preincubation of SHP2cd (150 nM, magenta circles) or C333P SHP2cd (150 nM, green circles) with the indicated concentrations of miconazole at 22 °C, the PTP activities of the enzymes were measured with pNPP (5 mM). DMSO concentration was 2.5%. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

had been treated with hydrogen peroxide. By contrast, Machado and co-workers observed only a C367–C459 disulfide bond, with no evidence of participation by C333, in the crystal structure of an oxidized SHP2 catalytic domain [24]. Although further inquiry will be needed to fully assess C333's function in SHP redox chemistry, particularly in a biological context, it does seem reasonable to surmise that the extra backdoor cysteine plays some role, if only complementary, in protecting the SHPs' catalytic cysteines from oxidation. If so, the discovery of C333 instability in the current study suggests that the SHPs have acquired augmented oxidative protection at the cost of decreased protein-fold stability during the course of evolution.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Anthony C. Bishop reports financial support was provided by National Institutes of Health.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2022.101370>.

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