Thioredoxin reductase 1 and NADPH directly protect protein tyrosine phosphatase 1B from inactivation during H$_2$O$_2$ exposure

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ABSTRACT

Regulation of growth factor signaling involves reversible inactivation of protein tyrosine phosphatases (PTPs) through the oxidation and reduction of their active site cysteine. However, there is limited mechanistic understanding of these redox events and their co-ordination in the presence of cellular antioxidant networks. Here, we investigated interactions between PTP1B and the peroxiredoxin 2 (Prx2)/thioredoxin 1 (Trx1)/thioredoxin reductase 1 (TrxR1) network. We found that Prx2 becomes oxidized in PDGF-treated fibroblasts, but only when TrxR1 has first been inhibited. Using purified proteins, we also found that PTP1B is relatively insensitive to inactivation by H$_2$O$_2$, but found no evidence for a relay mechanism in which Prx2 or Trx1 facilitates PTP1B oxidation. Instead, these proteins prevented PTP1B inactivation by H$_2$O$_2$. Intriguingly, we discovered that TrxR1/NADPH directly protects PTP1B from inactivation when present during the H$_2$O$_2$ exposure. This protection was dependent on the concentration of TrxR1 and independent of Trx1 and Prx2. The protection was blocked by auranofin and required an intact selenocysteine residue in TrxR1. This activity likely involves reduction of the sulfenic acid intermediate form of PTP1B by TrxR1 and is therefore distinct from the previously described reactivation of end-point oxidized PTP1B, which requires both Trx1 and TrxR1. The ability of TrxR1 to directly reduce an oxidized phosphatase is a novel activity that can help explain previously observed increases in PTP1B oxidation and PDGF receptor phosphorylation in TrxR1 knockout cells. TrxR1’s activity is therefore of potential relevance for understanding the mechanisms of...
INTRODUCTION

Protein tyrosine phosphatases (PTPs) are important regulators of receptor tyrosine kinase (RTK) signaling through dephosphorylation of tyrosine residues. Dysregulated RTK signaling pathways have been implicated in several diseases including cancer, atherosclerosis and diabetes (1-4). The activities of classical PTPs depend upon a low pH, Cys residue in the active site (1-3,5) that is susceptible to reversible inhibition by cellular oxidants produced following receptor stimulation (5,6). Reversible oxidation of PTPs is a well described contributor to growth factor signaling (5). The mechanism involves activation of membrane-bound NADPH oxidases (NOXs) (7), and possibly mitochondrial sites (8), to increase cytoplasmic superoxide and hydrogen peroxide (H$_2$O$_2$) levels with resultant PTP oxidation. Oxidation of PTPs by H$_2$O$_2$ can generate sulfenic, sulfinic (-SO$_2$) or sulfonic (-SO$_3$) acid forms. In PTP1B the sulfenic acid is a short lived intermediate (9,10) that converts to a sulfinylamide through a covalent bond with the peptide nitrogen of a juxtaposed serine residue (11-13). Rapid conversion from sulfenic acid to sulfinylamide is thought to protect the active site cysteine from further irreversible oxidation (9,11,12,14-16).

For PTP oxidation to be an effective control mechanism for RTK signaling, both inactivation and re-activation need to be carefully regulated, through selective oxidation and efficient reduction. To date, studies have mainly focused on understanding PTP oxidation, although there is still uncertainty about exact mechanisms. The initial oxidant is widely considered to be H$_2$O$_2$ yet it is not clear how PTPs, which in isolation react slowly with H$_2$O$_2$ (9) can be oxidized in cells containing peroxidases such as peroxiredoxins (Prxs) and glutathione peroxidases that are up to a million times more reactive (17). One mechanism could involve a redox relay with a Prx acting as a mediator of oxidant transfer. Such a mechanism has been described for Prx2-mediated formation of disulfide-linked STAT3 oligomers (18). Our first aim was to establish whether PTP1B oxidation could occur via a relay mechanism involving Prx2. We chose PTP1B because of its importance as a negative regulator of growth factor signaling. Oxidative inhibition of PTP1B is known to positively regulate RTK signaling via the platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptor pathways (8,19). Although PTP1B resides in the ER membrane, its catalytic domain faces the cytosol (20). Therefore, we focused on Prx2 as a major cytoplasmic Prx. Prx2 reacts readily with H$_2$O$_2$ and is recycled primarily by the Trx/TrxR system (21,22), and evidence for crosstalk between PTPs and Prx2 during PDGF signaling has been described (23).

Our second aim was to characterize reduction pathways that could regulate PTP1B activity. We and others have shown that fully oxidized PTP1B (presumably the sulfinylamide) can be reactivated by the Trx system (Trx1, TrxR1 and NADPH) (19,24-26), and that PTP1B oxidation and PDGF signaling is enhanced in TrxR1 knockout cells (24). Here we have used purified proteins to examine how components of the Prx2/Trx1/TrxR1 system affect the oxidation and reduction of PTP1B when present during exposure to H$_2$O$_2$. We found that the Prx2/Trx1/TrxR1 system inhibited inactivation of PTP1B by H$_2$O$_2$, with no evidence of a Prx2 relay system for PTP1B oxidation. Our investigation of the reductive mechanism showed that TrxR1 directly protects PTP1B against inactivation by H$_2$O$_2$, with the sulfenic acid of PTP1B. As far as we are aware, this would be the first reported example of TrxR1 reducing a protein sulfenic acid and hereby identifies a potential new mechanism for redox control of growth factor signaling pathways.

RESULTS

Stimulation of mouse embryonic fibroblasts by PDGF-BB induces Prx2 dimer formation in the presence of auranofin. PDGF stimulation has previously been shown to increase cellular oxidant production (27,28). H$_2$O$_2$ production has been detected with the specific biosensor HyPer, which was shown to undergo a continuous increase in fluorescence for at least 5 min following stimulation (29). As confirmation of this, we expressed HyPer in...
mouse embryonic fibroblasts (MEFs) expressing PDGFR-β, and observed that addition of the PDGF-BB ligand (which activates PDGFR-β) (30) caused a continuous increase in cellular fluorescence for at least 20 minutes that was significantly greater than in untreated cells (Fig. 1A & B). To determine if Prx2 becomes oxidized in this system, we measured its redox state at different times after PDGF-BB stimulation. In unstimulated cells, about half the Prx2 resolved in the reduced monomeric form and half as disulfide-linked dimers (Fig. 1C & D). This ratio did not change significantly in the 40 min following stimulation. The disulfide of Prx2 is recycled predominantly by the Trx system. When cells were pretreated with auranofin, a potent TrxR inhibitor (31,32), the basal level of Prx2 oxidation was unchanged. However PDGF-BB stimulation caused an increase in Prx2 dimers within 5 min and these remained almost completely oxidized (Fig. 1E & F). These findings suggest that PDGF receptor activation can induce oxidation of Prx2, but oxidized Prx2 does not accumulate because it is recycled by the Trx system.

**Recombinant reduced Prx2 protects PTP1B from inactivation by H$_2$O$_2$.** In theory, Prx2 could scavenge H$_2$O$_2$ and protect PTPs against oxidation, or promote oxidation via a redox relay system. To investigate the interactions of Prx2 with PTP1B, we prepared active recombinant human PTP1B and compared the effect of H$_2$O$_2$ on its activity in the absence and presence of Prx2. PTP1B undergoes different types of cleavage in mammalian tissues and when expressed in E. coli (33-35). Here we confirmed that PTP1B expressed in full length form in E. coli is cleaved upon lysis (34). We also expressed the isolated catalytic domain (26). The PTP1B cleaved variant was characterized as a ~50kDa band (tag and linker included) on SDS-PAGE and was used unless stated otherwise (Fig. 2A). Comparing the two PTP1B forms we found them to have similar activities. As expected from the measured rate constant (9), initial analyses using a chromogenic substrate, pNPP, showed that direct inactivation of PTP1B was slow and required exposure to 100 μM H$_2$O$_2$ for 30 min or to 1 mM H$_2$O$_2$ for 5 min for maximal effect (Fig. 2B). The effect of reduced Prx2 on PTP1B inactivation was initially examined using equimolar H$_2$O$_2$ and Prx2. With such conditions, the Prx2 should rapidly react with the H$_2$O$_2$, and if a relay mechanism operated it should result in more PTP1B inactivation. However, reduced Prx2 completely inhibited inactivation (Fig. 2C). We also tested whether oxidized Prx2 alone could inactivate PTP1B by disulfide exchange. There was no significant effect on activity (Fig. 2C). Non-reducing SDS-PAGE confirmed that Prx2 remained oxidized upon incubation with reduced PTP1B and no high molecular weight complexes between the two were observed (Fig. 2D). Thus, independent methods for monitoring oxidation of the two proteins showed no evidence of transfer of oxidizing equivalents from dimeric Prx2 to reduced PTP1B. Oxidized Prx1 was also tested for effects on PTP1B activity and showed no significant difference compared to controls (Fig. 2E).

**The Trx system together with Prx2 protects PTP1B from inactivation during exposure to H$_2$O$_2$.** To test if Prx2 could facilitate PTP1B oxidation while undergoing redox cycling, we treated PTP1B with excess H$_2$O$_2$ in the presence of Prx2 plus TrxR1/Trx/NADPH. All concentrations of Prx2 together with TrxR1/Trx1/NADPH showed clear protection, with no evidence of accelerated inactivation (Fig. 3A). Non-reducing SDS-PAGE confirmed that a proportion of Prx2 was initially oxidized to the disulfide at the 2-minute time point (dark arrow, Fig. 3B) then subsequently reduced by TrxR1/Trx1/NADPH at 20 and 30 minutes (lighter arrows, Fig. 3B). These results collectively suggest that Prx2 protects PTP1B by clearance of H$_2$O$_2$ with no evidence of facilitated oxidation by oxidized forms of Prx2.

**TrxR1 with NADPH alone protects PTP1B from inactivation during exposure to H$_2$O$_2$.** PTP activity can be regulated not only by oxidation but also by the rate of reduction of the reversibly oxidized protein. This is apparent in Fig. 3, where the PTP1B was reactivated once the Prx2 became reduced. Furthermore, with a higher TrxR concentration and the full Trx1/TrxR1/NADPH system, transient inactivation was not seen and the majority (80%) of the PTP1B activity was preserved after 30 min of H$_2$O$_2$ exposure (Fig. 4A). Surprisingly, whether or not Prx2 was present made no difference to the protective effect.

One explanation for PTP1B not being inactivated could be rapid consumption of the
added H$_2$O$_2$. This would be expected with Prx2 present, due to its well-characterized peroxidase activity, and indeed Prx2 with the full Trx system rapidly cleared H$_2$O$_2$ (Fig. 4B). However, the Trx system is inefficient at removing H$_2$O$_2$ (36) and no detectable H$_2$O$_2$ consumption was observed without Prx2 with any combinations of PTP1B and the Trx system (Fig. 4B).

Alternatively, the Trx system could reactivate the oxidized PTP1B. We first investigated this with PTP1B that had been pre-oxidized by H$_2$O$_2$, and confirmed our previous findings (24) that inactivation can be reversed by the complete Trx1/TrxR1/NADPH system (Fig. 4A). This required NADPH, and did not occur with TrxR1 in the absence of Trx1. However, with both the cleaved PTP1B protein (Fig. 4C and the catalytic domain (Fig. 4D), reactivation was slow, with only 30-35% recovery over 30-45 min. Under these conditions, DTT appeared to be more effective than the thioredoxin system. This difference was not observed in previous work (24), perhaps because a different substrate was used or the reaction was performed at a different temperature.

These results suggest that reactivation of the fully oxidized (sulfinylamide) form may be too slow to account for the protection of PTP1B during exposure to H$_2$O$_2$. Rather, they suggest that the Trx system continuously regenerates active PTP1B by reduction of an oxidation intermediate. This is most likely to be the sulfenic acid, which is the initial short-lived intermediate formed in the reaction of H$_2$O$_2$ with PTP1B (10-13).

Further experiments were performed to test how the Trx system protects PTP1B against H$_2$O$_2$-mediated inactivation. Intriguingly, TrxR1 in combination with NADPH protected equally efficiently as when Trx was present (Fig. 5A). These experiments were performed with the PTP1B cleaved variant, but results were similar with the catalytic domain (Fig. 5B). NADPH was required, and protection increased with increasing TrxR1 concentration (Fig. 5C). Therefore the observed protection against PTP1B oxidation is likely to involve a direct reaction of TrxR1 with PTP1B.

To see whether this mechanism applies generally to other PTPs, we examined human receptor-like density-enhanced protein tyrosine phosphatase-1 (DEP-1). The intracellular domain of recombinant GST-tagged human DEP-1 was purified and treated identically to PTP1B. DEP-1 was similar to PTP1B in its sensitivity to H$_2$O$_2$ (54% inactivation after 30 min exposure to 150 µM H$_2$O$_2$), but it was not significantly protected against inactivation by TrxR1 and NADPH (55% inactivation) (Fig. 5D). This implies a degree of specificity of TrxR1 for the reduction of PTP1B.

The active site selenocysteine residue of TrxR1 is indispensable for protecting PTP1B from H$_2$O$_2$. Further evidence that protection of PTP1B required active TrxR1 was obtained using auranofin, a potent inhibitor of TrxR1 that is presumed to interact with its active site Sec (37). Auranofin had no effect on PTP1B activity in the absence of H$_2$O$_2$ but abolished the protective effect of TrxR1 (Fig. 6A). Finally, we confirmed the requirement for Sec-dependent activity using TrxR1 mutants with the Sec mutated to either Cys or Ser (38). Measurement of PTP1B activity after exposure to H$_2$O$_2$ showed that the protective effect of wild type TrxR1 was lost in both mutants (Fig. 6B). Thus, the active site Sec is indispensable for TrxR1 to protect PTP1B against H$_2$O$_2$-mediated inactivation.

**DISCUSSION**

In the work presented here we have investigated regulatory mechanisms that influence both oxidation and reduction of PTP1B. Under the conditions used in this study, we found no evidence for Prx or Trx acting as redox relays that transfer oxidizing equivalents from H$_2$O$_2$ to PTP1B. Instead, the Trx system coupled with Prx2 protected PTP1B from oxidation. We also discovered a hitherto unknown direct reaction of TrxR1 with a PTP1B oxidation intermediate that protects against inactivation during exposure to H$_2$O$_2$. These findings have implications for understanding PTP1B regulation (Reaction 1, Figure 6C).

The prevailing view is that PTP oxidation occurs via H$_2$O$_2$ generated by NOX enzymes (7), although there are relatively few studies where this has been demonstrated unequivocally. Our finding that MEF cells expressing HyPer increased in fluorescence after stimulation with PDGF support other evidence (29) that H$_2$O$_2$ is indeed produced. Despite this, the reversibly oxidized form of the highly H$_2$O$_2$-reactive Prx2 was not significantly altered during stimulation unless TrxR1 was
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inhibited by auranofin. This implies that Prx2 is oxidized during PDGF stimulation but is efficiently recycled by the Trx system. Prx2 hyperoxidation was not directly analyzed however the decrease of monomer form upon auranofin treatment during stimulation suggest against formation of hyperoxidative forms.

As observed here and by others (9,26), oxidation of isolated PTP1B requires extended exposure to high concentrations of H₂O₂. Yet PTP1B is readily oxidized in cells (39) where Prx2 and other more reactive targets are present. A mechanism for facilitated oxidation would therefore seem essential, and a relay mechanism is an attractive concept (17,40-42). The observation that Prx2 turnover occurs during signaling raises the question of whether Prx2 acts as a relay protein. To answer this, we set up an in vitro system with protein concentrations estimated to reflect cellular levels (17). We found that reduced Prx2 with or without Trx and redox cycling protected PTP1B against H₂O₂ rather than enhancing inactivation. This, plus the lack of a detectable disulfide exchange between oxidized Prx2 and reduced PTP1B, is evidence against a redox relay. We cannot, however, exclude a relay mechanism via another sensor or if it requires an additional cellular interaction partner. An alternative mechanism for facilitated PTP oxidation might be that it is localized to where the oxidant source and PTP are in close proximity. In this case, co-localized Prx2 could prevent PTP inactivation by scavenging basal H₂O₂ (Reaction 2 in Figure 6C), and limit global oxidation of cellular proteins. Protection of PTP1B could be overcome if receptor activation resulted in localized inactivation of the Prx, either through oxidation or by peroxidase activity being decreased due to phosphorylation (43,44). This model has been suggested as an explanation for increased PDGF-dependent phosphorylation in Prx2-deficient cells with corresponding decrease in total phosphatase activity of membrane fractions (23). However, it is still unlikely that oxidation could be due to freely diffusible H₂O₂ (45).

While oxidative inactivation of PTPs is one mechanism for promulgating RTK pathways, PTPs also need to be recycled as a part of signal regulation. For PTP1B, there is good evidence from our studies of Txnrd1−/− MEF cells that TrxR1 is involved in recycling (22). We found increased basal levels of PTP1B oxidation in these cells compared with wild type cells, and PDGF-BB caused relatively greater phosphorylation of the PTP1B site of the PDGF receptor and cell proliferation. The phosphorylation changes were mimicked by adding auranofin. Recycling of PTP1B could occur either by reducing the fully oxidized sulvenylamide end product, or in a more dynamic process involving reduction of the sulfenic acid intermediate. We and others (19,24-26) have shown that fully oxidized PTP1B can be at least partially reactivated by the full thioredoxin system (Reaction 3, Figure 6C). Here we confirmed these findings with both the cleaved PTP1B protein used in this study and the isolated catalytic domain used previously.

In cells during growth-factor receptor activation, reductant systems will be present while PTPs are exposed to H₂O₂ and would have the potential to react with the sulfenic acid. When we simulated this situation by exposing reduced PTP1B to H₂O₂ together with components of the Trx system, we observed highly efficient protection of PTP1B from H₂O₂-mediated inactivation. This could not be accounted for by the slower regeneration of the end product of oxidation. Neither was it due to consumption of H₂O₂ by TrxR1. TrxR1 does react slowly with H₂O₂ and indeed calculations from the kinetic data (36) suggest a rate constant of ~600 M⁻¹ s⁻¹. This is greater than the value of 20 M⁻¹ s⁻¹ for PTP1B (46). However, the TrxR1 rate is not fast enough to consume more than a few micromolar H₂O₂ during the experiment with PTP1B. Most strikingly, protection was provided by TrxR1 activity with no requirement for Trx. Further characterization revealed that protection requires the active site Sec, as it was blocked by the TrxR inhibitor auranofin and lost when the Sec was replaced by Cys or Ser. As TrxR1 in the absence of Trx was unable to reactivate PTP1B after its conversion to the sulvenylamide form, a likely explanation of our findings is that the Sec-containing active site of TrxR1 directly reduces the sulfenic acid of PTP1B (Reaction 1, Figure 6C). Notably, as seen in Fig. 6A and 6B, a gradual loss of PTP1B activity is still seen over time in the presence of TrxR1, which should likely be due to a minor fraction of PTP1B continuously forming the sulvenylamide form, which cannot be reactivated by TrxR1 alone. As far as we are aware, direct reduction of a protein sulfenic
acid is a role that has not previously been described for TrxR1. Our results with DEP-1 indicate that this is not a universal mechanism. However, reduction of the sulfinic acid has important implications for TrxR1 as a regulator of PTP1B activity.

TrxR1 could in theory regulate PTP1B activity during cell signaling by several mechanisms (Figure 6C): i) by supporting the \( \text{H}_2\text{O}_2 \) scavenging activity of Prx; ii) by acting with Trx1 to recycle PTP1B once oxidized; or iii) by directly intercepting the sulfinic acid intermediate. These modes of regulation could operate in parallel with increased oxidant production due to NOX activation. Further control could be exerted by downregulation of the reductive capacity of TrxR, for example through NADPH depletion or reversible inactivation by nitrosylation (47). TrxR activity with PTP1B would also be decreased if its preferred substrate, oxidized Trx, increased due to an increasing demand of Trx in reduction of other substrates, such as oxidized Prx2 (Reaction 2, Fig 6C). This could enable oxidized PTP1B to accumulate. These mechanisms involving TrxR1 are all potential contributors to the enhanced PTP1B inactivation and growth factor signaling that has been observed in Trxrd1-/- cells. We previously speculated that the phenotype of the knockout cells could be explained by the reduction of fully oxidized PTP1B by the complete Trx system (24). However, subsequent findings that Trx1 is kept reduced through the GSH system in these cells (48) support a mechanism such as described here for sulfinic acid reduction, where only TrxR1 is required.

**EXPERIMENTAL PROCEDURES**

**Preparation of recombinant proteins.** cDNA encoding recombinant 6xhistidine-tagged full length human PTP1B (a kind gift from Professor T.C. Meng, Academia Sinica) was subcloned into pET28a vector (Novagen) by PCR with primers 5'-GCGGAATTCATCGAAGGTCGTATGGAGAAGGGATTTCGAG-3' and 5'-GCCGACCATATGTTGCGTTGAA-3' and EcoR1 and Sal1 restriction sites. Expression, purification and removal of tag were performed using previously described protocol (38). His-tagged PTP1B and Prx2 were expressed and purified and the His-tag of Prx2 was cleaved off as previously described (49), except that PTP1B was purified in the presence of 5 mM β-mercaptoethanol to maintain the protein in its reduced form (49). The intracellular domain containing the catalytic center of DEP-1 (a kind gift from Professor F. Böhmer, Institute for Molecular Cell Biology) was expressed and purified as previously described (50). Recombinant human Trx1 and rat TrxR1 wild type and mutant proteins were expressed and purified as described previously (38). His-tagged PTP1B cleaved form, PTP1B catalytic domain and DEP-1 resolved by SDS-PAGE corresponded to molecular masses of ~50 kDa, ~37 kDa (Figure S1) and ~70 kDa respectively (data not shown). Prior to all experiments Prx2 preparations were reduced with 10 mM DTT and Prx2, PTP1B and DEP-1 were all subjected to buffer exchange to remove reductant using Bio-Gel P-6DG-Gel (BioRad Cat #150-0738) columns (51). Protein concentrations were determined by Direct Detect (Millipore).

**PTP activity assay.** PTP activity was determined using 15mM chromogenic substrate 4-nitrophenyl phosphate (pNPP) (P4744-1G, Sigma-Aldrich) as described previously (52). The release of 4-nitrophenol was measured at 410 nm at 22°C using a Varioskan Flash plate reader (Thermo Scientific). Reduced PTP1B (600 nM, 32.5 μg/ml) or DEP-1 (80 nM, 5.6 μg/ml) was pre-incubated for 20 min in 20 mM HEPES, 100 mM NaCl buffer pH 7.4 containing 0.1 mM DTPA, 0.05% bovine serum albumin (BSA), 1 mM sodium azide, with indicated concentrations of Trx1, TrxR1, NADPH (N7505-100MG, Sigma-Aldrich) and Prx2. Sodium azide was used to inhibit any trace amounts of catalase. Control PTP1B and DEP-1 activities gave a substrate turnover of (2.6-17, median 7.1) and (30-33) nmole/min/µg, respectively. Variations in activity were observed between different batches of PTP purifications.
SDS-PAGE analyses. The oxidation state of recombinant Prx2 was determined by separation of monomers and disulfide-bonded dimers by non-reducing SDS-PAGE (12% gel) with subsequent silver or Coomassie staining. Prx2 oxidation state in MEF cells was determined after PDGF-BB stimulation, with and without pretreatment of the cells with auranofin (1 µM for 1 h). Reduced thiols were blocked by adding 10 mM NEM in buffer (50 mM Tris pH 7.4 / 150 mM NaCl) and 1 µg/ml catalase to the intact cells, a procedure we have established to provide effective blocking (53). Cells were then trypsinized, washed once with buffer and lysed with buffer + 1% NP40 (Pierce) and Complete protease inhibitors (Roche). Lysates were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), blocked with 5% milk in Tris-buffered saline and immunoblotted for Prx2 (Sigma R8656). Prx2 dimer to total Prx2 ratios were quantified using UV Band analysis software (UVITEC).

Treatment of PTP1B with H$_2$O$_2$. Buffer-exchanged reduced PTP1B was exposed to H$_2$O$_2$ and different components of the Trx system at indicated time points followed by addition of substrate and measurement of activity. We initially observed that incubation of PTP1B alone resulted in some time-dependent inactivation that was partially prevented in the presence of BSA, so BSA (0.05%) was added to the buffer. The activity after each H$_2$O$_2$ treatment was related to the activity of untreated PTP1B incubated for the same time. A 0-24% loss of activity was seen in control conditions at 30 min.

Re-activation of H$_2$O$_2$-treated PTP1B. Re-activation was performed using H$_2$O$_2$-inactivated PTP1B. Reduced PTP1B was first buffer-exchanged into re-activation buffer as previously (26), and inactivated with 100 µM or 1 mM H$_2$O$_2$ for 5 min. Excess H$_2$O$_2$ was removed by adding 20 µg/ml catalase before re-activation was performed by adding components of the Trx system as indicated.

H$_2$O$_2$ measurement. Combinations of PTP1B, Trx1, TrxR1, NADPH and Prx2 (in 20 mM HEPES, 0.05 % BSA, 1mM sodium azide pH 7.4) were treated with 100 µM H$_2$O$_2$ and the loss of H$_2$O$_2$ monitored using the ferrous oxidation of xylenol orange (FOX) assay (54).

Cell culture and transient transfection with HyPer. Mouse embryonic fibroblasts (MEFs) (Walter and Elisa Hall Institute) were cultured in DMEM + 10% (vol/vol) fetal bovine serum (FBS), 2 mM L-glutamine, penicillin and streptomycin. Cells were stimulated with PDGF-BB (R&D Systems #220-BB) after overnight serum starvation in DMEM + 1% FBS.

Transient transfection of MEF cells with HyPer. MEF cells were seeded into 6-well plates. Transfections were with HyPer-Cyto plasmid (a kind gift from Belousov V, Russian Academy of Sciences) (6.5 µg) mixed with 0.5 ml OptiMEM reduced serum medium and 25 µl of 1 mg/ml polyethylenimine (linear, MW 25,000, Polysciences Inc., Warrington, PA, USA). The transfection mixture was incubated for 30 min at room temperature before addition to MEF cells in antibiotic-free medium. The medium was changed the following morning.

Live cell fluorescence imaging. Live cell imaging was performed with an Olympus IX81 Motorized Inverted Microscope. pHyper-Cyto transfected MEF cells were starved overnight and then stimulated with PDGF-BB. Hyper probe fluorescence was monitored at 1 min intervals over 20 min using a FITC channel at 495 nm / 519 nm (55). Quantifications were performed using ImageJ as previously described (56).

Statistical analyses. Analyses of data were performed using one-way ANOVA followed by Tukey post hoc tests for multiple comparisons. Analyses of HyperCyto derived fluorescence was analyzed using students paired t-test.
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CONFLICT OF INTEREST:
The authors declare that there are no competing interests.

AUTHOR CONTRIBUTIONS:
M.D. has performed all experiments, analysis of data and contributed writing the paper. P.E.P has expressed Prx2 and contributed to cloning and expression of PTP1B as well as writing the manuscript, Q.C. has expressed, purified, TrxR1, Serine/cysteine, variants and Trx1. J.F. has provided intellectual input. A.O. has provided intellectual input. E.S.JA. designed experiments, analyzed data, provided essential intellectual input and contributed to writing the paper. M.B.H. designed experiments, analyzed data, provided intellectual input and contributed to writing the paper. C.C.W. was overall responsible for the project and contributed to design of experiments, analyzed data, provided intellectual input and wrote the paper.

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**FIGURE LEGENDS**

Fig. 1. *Prx2 dimer formation is induced by PDGF-BB treatment in MEFs cells pretreated with auranofin*. **A**: Representative images of MEF cells expressing cytosolic HyPer after 0 and 20 min without treatment (Control), or treatment with 100 ng/ml PDGF-BB. **B**: Change in fluorescence over 20 min in untreated or PDGF-BB stimulated Hyper-expressing MEF cells. Data from 19 transfected cells analyzed in 5 different experiments (mean ±SE *P < 0.05). **C**: Prx2 immunoblot of non-reducing SDS-PAGE-resolved lysates from MEF cells treated with 50 ng/ml PDGF-BB for the indicated times. **D**: Quantification of Prx2 dimers (percentage of total Prx2) from densitometry analyses of blots represented in C (n=7). **E**: Immunoblot as in C of lysates from cells pretreated for 1 h with the TrxR1 inhibitor auranofin (1 µM) before PDGF-BB treatment. **F**: Quantitation of Prx2 dimers in the auranofin-treated cells determined as in D.

Fig. 2. *Reduced Prx2 protects PTP1B from inactivation by \( \text{H}_2\text{O}_2 \).* **A**: Analysis of PTP1B His-tagged cleaved variant and PTP1B catalytic domain on SDS-PAGE stained with coomassie blue. Purity was ~72% and ~99% respectively as determined by densitometry using Image J. Side by side comparison of activity of reduced PTP1B cleaved variant and PTP1B catalytic domain showed a mean substrate turnover of 269 and 355 min\(^{-1}\) respectively (n=3). **B**: \( \text{H}_2\text{O}_2 \)-dependent inactivation of recombinant PTP1B. Reduced PTP1B (600 nM) was exposed to 100 µM or 1 mM \( \text{H}_2\text{O}_2 \) for designated times, and subsequently analyzed for PTP activity using a pNPP substrate (n=3, ±SEM, *P < 0.05). **C**: PTP1B activities are expressed as percentages of untreated controls. **D**: Activity of PTP1B following treatment with \( \text{H}_2\text{O}_2 \) and reduced or oxidized Prx2. Fully reduced PTP1B (600 nM) was incubated for 30 min with 20 µM reduced Prx2 and 20 µM \( \text{H}_2\text{O}_2 \), or reduced or oxidized Prx alone, then assayed for PTP activity (n=3, ±SEM *P < 0.05, \( \text{H}_2\text{O}_2 \) treated compared to controls). **E**: Activity of PTP1B following treatment with \( \text{H}_2\text{O}_2 \) or oxidized Prx1. Equimolar reduced PTP1B (150 nM) and oxidized Prx2 were incubated for 30 min and analyzed by non-reducing SDS-PAGE. A silver stained gel (representative from 3 independent experiments) shows the positions of individual proteins as well as reduced Prx2 for comparison. The lower and upper oxidized Prx2 bands correspond to dimers containing one or two disulfides respectively. **F**: Activity of PTP1B following treatment with \( \text{H}_2\text{O}_2 \) or oxidized Prx1. Fully reduced PTP1B (600 nM) was incubated for 30 min with 10 µM \( \text{H}_2\text{O}_2 \), or 10 µM oxidized Prx alone, then assayed for PTP activity (n=3, ±SEM *P < 0.05, \( \text{H}_2\text{O}_2 \) treated compared to controls).
Fig. 3. Prx2 redox cycling with the Trx system protects PTP1B against inactivation during exposure to H$_2$O$_2$. A: Effects of Prx2 and Trx system components for inactivation of PTP1B with H$_2$O$_2$. Reduced PTP1B catalytic domain (600 nM) was pre-incubated for 30 min, with 2µM Trx1, 50nM TrxR1 (specific activity 17 U/mg), 200 µM NADPH, and 0-20 µM Prx2. H$_2$O$_2$ (100 µM) was then added and samples were taken at 5, 15 and 30 min for measurement of PTP activity (n=3, ±SEM *P < 0.05). B: Parallel analysis of Prx2 redox status from experiment (A). Aliquots were taken from assay (A) after 2, 20 and 30 min of H$_2$O$_2$ treatment and analyzed by non-reducing SDS-PAGE. A coomassie stained gel (representative from 3 independent experiments) shows the positions of reduced and oxidized Prx2, the latter (arrow) showing two Prx2 bands corresponding to one or two disulfides.

Fig. 4. The Trx system reverses H$_2$O$_2$ inactivated PTP1B. A: Reduced PTP1B (600 nM) was pre-incubated for 30 min, either alone or with 2µM Trx1, 0.5 µM TrxR1 (specific activity 9.75 U/mg) and 200 µM NADPH, with or without Prx2 (10 µM). H$_2$O$_2$ (100 µM) was then added and samples were taken at 5, 15 and 30 min for measurement of PTP activity (n=3, ±SE *P < 0.05). B: H$_2$O$_2$ consumption by PTP1B and components of the Trx/Prx2 system. Combinations of Trx1 (2 µM), TrxR1 (0.5 µM, 18U/mg), Prx2 (10 µM) and NADPH (200 µM), with and without reduced PTP1B (600 nM) were treated with 100 µM H$_2$O$_2$ at 22°C and concentrations of H$_2$O$_2$ at indicated time points were determined by FOX assay (n=3, ±SEM *P < 0.05). C: Re-activation of H$_2$O$_2$-inactivated PTP1B (cleaved form). Reduced PTP1B was treated with 1 mM H$_2$O$_2$ for 5 min, then with catalase to remove residual H$_2$O$_2$ and reactivated with 10 mM DTT or with TrxR1 (2.5 µM) (specific activity 18 U/mg) and NADPH (1 mM) with or without Trx1 (10 µM). After 45 min at 22°C, samples were analyzed for PTP activity (n=3, ±SEM, *P < 0.05). D: Re-activation of H$_2$O$_2$-inactivated PTP1B catalytic domain. Reduced PTP1B was treated with 1 mM H$_2$O$_2$ for 5 min, then with catalase and reactivated with 10 mM DTT or with TrxR1 (2.5 µM) (specific activity 22 U/mg), NADPH (300 µM) and Trx1 (10 µM). After 5,10 and 40 min at 22°C, samples were analyzed for PTP activity (n=3, ±SEM *P < 0.05).

Fig. 5. TrxR1 activity protects PTP1B inactivation during exposure to H$_2$O$_2$. A: Protection of PTP1B from H$_2$O$_2$ requires only TrxR1 and NADPH. PTP1B was treated with H$_2$O$_2$ in the presence of various components of the Trx system and assayed for PTP activity as in Figure 3B. Rates of inactivation with Trx1/TrxR1 or TrxR1 without NADPH were indistinguishable from that with PTP1B alone (in Figure 3B) (n=3, ±SEM *P < 0.05). B: Reduced PTP1B cleaved and PTP1B catalytic domain variant (600 nM) was treated with the indicated concentrations of TrxR1 (0.5 µM) and 200 µM NADPH and exposed to 100 µM H$_2$O$_2$ for 5, 15 and 30 min (n=3, ±SEM *P < 0.05). C: Concentration-dependent protection of PTP1B activity by TrxR1. Reduced PTP1B (600 nM) was treated with the indicated concentrations of TrxR1 and 200 µM NADPH and exposed to 100 µM H$_2$O$_2$ for 5 min (n=3, ±SEM *P < 0.05). D: DEP-1 was treated with H$_2$O$_2$ in the presence of TrxR1 and NADPH and assayed for PTP activity as in Figure 4B. Reduced DEP-1 (80 nM) was treated with the indicated concentrations of TrxR1 (0.5 µM) and 200 µM NADPH and exposed to 150 µM H$_2$O$_2$ for 5 min (n=3, ±SEM *P < 0.05).

Fig. 6. The active site selenocysteine residue of TrxR1 is indispensable for protection of PTP1B from H$_2$O$_2$-mediated inactivation. A: Inhibition of TrxR1 protection by auranofin. Reduced PTP1B (600 nM) was treated with or without TrxR1 (0.5 µM, 9.75U/mg), NADPH (200 µM) and auranofin (AU) (1 µM). PTP activity was measured at indicated times after addition of 100 µM H$_2$O$_2$ (n=3, ±SEM *P < 0.05). B: Lack of protection by active site mutants of TrxR1. Reduced PTP1B was exposed to 100 µM H$_2$O$_2$ in the presence of wild-type TrxR1 (18U/mg) or variants where the active site SelCys was mutated to Cys (TrxR1Cys) or Ser (TrxR1Ser), then assayed for PTP activity as in A (n=3, ±SEM *P < 0.05). C: Schematic illustration of how the redox activity of the Trx/TrxR/Prx system could regulate PTP1B activity. The bold arrows show the reaction of TrxR1 (green) with the sulfenic acid of PTP1B that we propose to explain protection of PTP1B activity (blue) by TrxR1 as observed in this study (reaction 1). Additional activities of the Trx system in relation to PTP regulation, as previously described in the literature, are schematically shown in grey. The full scheme is discussed in the text.
TrxR1 protects PTP1B from inactivation by H$_2$O$_2$

Fig. 1

A

Control

PDGF-BB

min

B

C

Dimer

Monomer

min 0 5 10 20 40

D

E

min 0 5 10 20 40

F

% increase in fluorescence

% Prx2 dimer

*
TrxR1 protects PTP1B from inactivation by H$_2$O$_2$

Fig. 2

A

B

C

D

E

PTP1B activity % of control

PTP1B reduced
Peroxidas-2
Peroxidas-2 monomer

PTP1B
PTP1B + H$_2$O$_2$
PTP1B + Peroxidas-2
PTP1B + Peroxidas-2 monomer

PTP1B activity % of control

100µM H$_2$O$_2$
1mM H$_2$O$_2$

PTP1B cleaved
PTP1B catalytic
TrxR1 protects PTP1B from inactivation by H$_2$O$_2$
TrxR1 protects PTP1B from inactivation by H₂O₂

Fig. 4

A

B

C

D

PTP1B activity (% of control)

0 10 20 30

time (min)

120

+TrxR1+NADPH+Trx1+Prx2
+TrxR1+NADPH+Trx1
Reduced PTP1B

PTP1B reduced
PTP1B + TrxR1 + NADPH
PTP1B + TrxR1 + Trx1 + NADPH
PTP1B + TrxR1 + Trx1 + Prx2 + NADPH
TrxR1 + NADPH
TrxR1 + Trx1 + NADPH

H₂O₂ (μM)

0 20 40 60 80 100

time (min)

120

PTP1B activity (% of control)

0 20 40 60 80 100

H₂O₂

+DIT

PTP1B reduced control
PTP1B oxidized control
+TrxR1 + NADPH + Trx1
+DIT

PTP1B activity (nmol/mg/min)

0 100 200 300 400 500

min
TrxR1 protects PTP1B from inactivation by H₂O₂
TrxR1 protects PTP1B from inactivation by H$_2$O$_2$
Thioredoxin reductase 1 and NADPH directly protect protein tyrosine phosphatase IB from inactivation during H$_2$O$_2$ exposure
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