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Differential Oxidation of Protein-tyrosine Phosphatases*

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Oxidation is emerging as an important regulatory mechanism of protein-tyrosine phosphatases (PTPs). Here we report that PTPs are differentially oxidized, and we provide evidence for the underlying mechanism. The membrane-proximal RPTPα-D1 was catalytically active but not readily oxidized as assessed by immunoprobing with an antibody that recognized oxidized catalytic site cysteines in PTPs (oxPTPs). In contrast, the membrane-distal RPTPα-D2, a poor PTP, was readily oxidized. Oxidized catalytic site cysteines in PTP immunoprobing and mass spectrometry demonstrated that mutation of two residues in the Tyr(P) loop and the WPD loop that reverse catalytic activity of RPTPα-D1 and RPTPα-D2 also reversed oxidizability, suggesting that oxidizability and catalytic activity are coupled. However, catalytically active PTP1B and LAR-D1 were readily oxidized. Oxidizability was strongly dependent on pH, indicating that the microenvironment of the catalytic cysteine has an important role. Crystal structures of PTP domains demonstrated that the orientation of the absolutely conserved PTP loop arginine correlates with oxidizability of PTPs, and consistently, RPTPμ-D1, with a similar conformation as RPTPα-D1, was not readily oxidized. In conclusion, PTPs are differentially oxidized at physiological pH and H_2O_2 concentrations, and the PTP loop arginine is an important determinant for susceptibility to oxidation.

Phosphorylation of proteins on tyrosine residues has an important role in many cellular processes like proliferation, differentiation, and migration. Tyrosine phosphorylation is mediated by the balanced action of protein-tyrosine kinases and protein-tyrosine phosphatases (PTPs)[1] (1). Not only protein-tyrosine kinases but also PTPs have important roles in diseases, including cancer and diabetes (2–5). The human genome encodes 103 cysteine-based PTP family members, of which 38 are strictly phosphotyrosine-specific (4, 5). These "classical" PTPs are subdivided into transmembrane, receptor PTPs (RPTPs) (21 genes) and intracellular, nonreceptor PTPs (17 genes). The cytoplasmic PTPs encode a single PTP domain, whereas most RPTPs contain two catalytic domains. The membrane-proximal domain (D1) contains most catalytic activity (6, 7), whereas the membrane-distal domain (D2) has a regulatory function (8). The PTPs, including the RPTP-D2s, are highly conserved in sequence and three-dimensional structure (9–11). Only two amino acid residues that are conserved in all active PTPs, but not in RPTP-D2s, are responsible for the lack of catalytic activity in RPTP-D2s, because mutation of these residues renders RPTP-D2s active (10, 12, 13).

Relatively little is known about the regulation of PTPs. Reversible oxidation of the absolutely conserved catalytic site cysteine that is essential for catalysis (14) is emerging as an important regulatory mechanism (15). The catalytic cysteines are susceptible to oxidation because of their low pK_a (16, 17). Oxidation of cysteine residues to sulfenic acid is reversible, whereas further oxidation to sulfinic (doubly oxidized) or sulfonic (triply oxidized) forms is irreversible (18). Oxidized PTP1B shows a newly identified bond, termed cyclic sulfenamide, in which the sulfur of the cysteine is covalently linked to the main chain nitrogen of the neighboring serine (19, 20). Cyclic sulfenamide, like sulfenic acid, can be reduced by thiols, and importantly, it suppresses oxidation to sulfinic and sulfonic acids.

There are many physiological stimuli that induce reactive oxygen species (ROS) production. For instance, ROS are produced in response to stimuli like UV light, growth factors, and insulin, leading to inactivation of PTPs (21–26). ROS-induced inhibition of PTP activity may be essential for stimulus-induced signaling. For instance, platelet-derived growth factor induces oxidation of Shp2, and *N*-acetyl cysteine, a ROS quencher, blocks Shp2 oxidation and reduces platelet-derived growth factor signaling (25). Interestingly, Reynolds *et al.* (27) demonstrated in a mathematical model that epidermal growth factor-induced epidermal growth factor receptor activation, together with ROS-mediated inactivation of PTPs is sufficient to cause lateral signal propagation.

PTPs are emerging as important redox sensors in cells. Recently, we found that RPTPα is regulated by oxidation in an unexpected way. Whereas RPTPα-D1 contains most of the catalytic activity, it appears that RPTPα-D2 is much more sensitive to oxidation than RPTPα-D1 *in vitro* and in cells in response to UV irradiation (28). Yet RPTPα is inactivated in

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[1] The abbreviations used are: PTP, protein-tyrosine phosphatase; RPTP, receptor PTP; D1, membrane-proximal PTP domain; D2, membrane-distal PTP domain; LAR, leukocyte common antigen related; ROS, reactive oxygen species; GST, glutathione *S*-transferase; DTT, dithiothreitol; MALDI-ToF, matrix-assisted laser desorption ionization time-of-flight; oxPTP antibody, antibody that recognizes oxidized catalytic site cysteines of PTPs; MES, 4-morpholineethanesulfonic acid.

response to H_2O_2 treatment, because RPTPα-D2 undergoes a conformational change in response to oxidation, leading to stabilization of an inactive dimeric conformation (8, 29). Mutation of the catalytic cysteine in RPTPα-D2 renders RPTPα less sensitive to oxidation, in that H_2O_2 and UV treatment of living cells do not lead to complete inactivation of RPTPα-C723S, whereas wild type RPTPα is inactivated completely (8, 28).

In the present study, we investigated differential oxidation of PTPs. oxPTP immunoprobing and matrix-assisted laser desorption ionization time-of-flight (MALDI-ToF) mass spectrometry indicated that the mutations in the Tyr(P) loop and the WPD loop that are responsible for the difference in catalytic activity between RPTPα-D1 and RPTPα-D2 were also involved in the difference in oxidizability. Furthermore, we demonstrate that PTP1B and LAR-D1 were highly oxidizable. The susceptibility of PTPs to oxidation was strongly dependent on pH. Comparison of the crystal structures suggested that the orientation of the PTP loop arginine is important for the oxidizability of PTPs. Taken together, the subtle differences in the microenvironment of the catalytic cysteines determine the oxidizability of PTPs.

MATERIALS AND METHODS

Constructs—pGEX-based bacterial expression vectors encoding GST fusion proteins of RPTPα-D1 contained the region from amino acids 167 to 503 and RPTPα-D2 from amino acids 504 to 793 (30). D2-E690D, D2-V555Y,and D2-E690D/V555Y have been described (13). RPTPα-D1-Y262V and D1-D401E were made by site-directed mutagenesis and verified by sequencing. The pGEX construct encoding zebrafish PTP1B (residues 1–282) has been described (31). pGEX-LAR-D1 and pGEX-LAR-D2 encoded human LAR residues 1275–1608 and 1609–1897, respectively. pGEX-RPTPμ-D1 encoded human RPTPμ residues 765–1165. These constructs were derived by PCR and verified by sequencing.

Analysis of Oxidized PTPs Using the oxPTP Antibody—Reversible oxidation was assayed using an antibody, oxPTP, that specifically recognizes the sulfonic acid form of catalytic site cysteines of PTPs (28, 32). As outlined in Fig. 1*A*, following treatment, the sample is alkylated to protect reduced cysteines. Subsequently, the sample is treated with dithiothreitol (DTT) to reduce reversibly oxidized cysteines and treated with pervanadate to convert reduced cysteines to the sulfonic acid form. Finally, the sulfonic acid form is detected using the oxPTP antibody by immunoblotting. The procedure has been described in detail (28). Briefly, GST fusion proteins, purified using standard procedures, were bound to glutathione beads. All of the incubations and washes were done with 20 mM Tris, pH 7.5, unless otherwise stated. The proteins were reduced for 30 min in 10 mM DTT. The beads were washed twice and were incubated with varying concentrations of H_2O_2 for 20 min. Immediately after oxidation proteins were alkylated, using 40 mM iodoacetic acid for 30 min. The proteins were washed extensively, reduced with 10 mM DTT, washed twice, and incubated for 1 h with 0.1 mM pervanadate (1 mM pervanadate, 1 mM Na_3VO_4 mixed with 5 mM H_2O_2, final concentrations, and left for at least 5 min at room temperature) to convert reduced cysteines irreversibly into the sulfonic acid form. Laemmli sample buffer was added, and the samples were boiled for 5 min and run on a 10% SDS-polyacrylamide gel (5 μg of protein/lane). The samples were transferred to polyvinylidene difluoride membrane by semi-dry blotting. For detection with oxPTP antibody, the blots were blocked in 0.1% bovine serum albumin, 0.1% Tween in 50 mM Tris, pH 7.5, 150 mM NaCl for 1 h or overnight and incubated with oxPTP antibody (1.5 h at room temperature or overnight at 4 °C). Subsequently, horseradish peroxidase-coupled goat-anti-rabbit (1:10,000; BD Pharmigen) was used, and the antibody signals were visualized by ECL. In some cases, the membranes were stripped and reprobed with anti-RPTPα antibody (30) and subsequently developed using ECL.

Phosphatase Assays—Fusion proteins were bound to GST beads in 20 mM Tris-HCl, pH 7.5, 10 mM DTT. The beads were incubated with varying concentrations of H_2O_2 for 20 min at pH 7.5 and then directly incubated in 20 mM MES buffer, pH 6.0, 1 mM EDTA, 150 mM NaCl, 10 mM *p*-nitrophenylphosphate for 45 min at 30 °C. The reactions were quenched with 0.4 M NaOH, and optical density was measured with a spectrophotometer at 440 nm (wavelength).

MALDI-ToF Mass Spectrometry—All of the reactions and washing steps were performed in 20 mM Tris-HCl, pH 7.5. GST fusion proteins of RPTPα-D2 and mutated RPTPα-D2 (V555Y, E690D, and V555Y/E690D) were immobilized on glutathione-Sepharose beads and incubated with 10 mM DTT. The beads were washed and incubated with H_2O_2 for 20 min at room temperature. Subsequently, the beads were treated with 100 mM iodoacetamide for 30 min at room temperature to derivatize the unaffected cysteines. Finally, the beads were washed and incubated with 10 mM DTT for 10 min to reduce singly oxidized cysteines. The fusion protein was cleaved off by thrombin protease (4 h at room temperature), and the protein of interest was subjected to overnight tryptic digestion. For desalting, the peptides were adsorbed on C_{18} ZipTips (Millipore) and washed with 0.1% trifluoroacetic acid. The peptides retained on the columns were eluted using α-cyano-4-hydroxycinnamic acid (10 mg/ml) in 10% acetonitrile, 0.1% trifluoroacetic acid. Peptide mass fingerprint spectra were recorded on a MALDI-ToF Voyager DE-STR (Applied Biosystems) mass spectrometer operated in positive ion Reflectron mode. After time-delayed extraction, the ions were accelerated to 20 kV for ToF mass spectrometric analysis. A total of 150 shots were acquired, and the signal was averaged per spectrum. MALDI-ToF spectra were analyzed using the Applied Biosystems Data Explorer software.

RESULTS

Differential Oxidation of RPTPα-D1 and RPTPα-D2—We investigated oxidation of PTPs using the oxPTP antibody (Fig. 1*A*). Consistent with a previous report (28), RPTPα-D1 was oxidized at 250 μM H_2O_2 but not at lower concentrations (Fig. 1*B*), and RPTPα-D2 was already detectably oxidized at the lowest concentration used (31 μM H_2O_2) (Fig. 1*C*). The negative control in our oxPTP experiments represents the fully reduced PTP, achieved by treatment with 10 mM DTT for 20 min. For the positive control, the alkylation step was omitted, leading to complete triple oxidation of the catalytic cysteine upon pervanadate treatment (Fig. 1).

In parallel with the oxPTP immunoprobing experiments, we performed PTP activity assays using *p*-nitrophenylphosphate as a substrate. RPTPα-D1 retained much of its activity at low concentrations of H_2O_2 (31 μM) (Fig. 1*D*). RPTPα-D2, although much less active than D1, is more susceptible to H_2O_2 than RPTPα-D1 and is already inactivated at low concentrations of H_2O_2 (31 μM; Fig. 1*D*). Together, these results show that RPTPα-D2 is more susceptible to oxidation than RPTPα-D1 as detected by the oxPTP antibody and by PTP activity assays.

Two Residues Determine the Difference between RPTPα-D1 and RPTPα-D2—Previously, we have shown that the difference in catalytic activity between RPTPα-D1 and RPTPα-D2 is caused by two residues in the Tyr(P) loop (Tyr^{262} in D1 and Val^{555} in D2) and WPD loop (Asp^{401} in D1 and Glu^{690} in D2) that are always conserved in catalytically active PTPs and never in inactive RPTP-D2s. The single mutants in RPTPα-D2, E690D and V555Y, regain some of their activity, and the double mutant V555Y/E690D is as active as RPTPα-D1 (12, 13, 33). These mutants were tested for their susceptibility to H_2O_2 using the oxPTP antibody. Whereas wild type RPTPα-D2 was oxidized at 31 μM H_2O_2, RPTPα-D2-V555Y was only oxidized at 125 μM H_2O_2 and above (Fig. 2*A*). Even higher levels of H_2O_2 were required for RPTPα-D2-E690D oxidation (Fig. 2*A*). Moreover, RPTPα-D2-V555Y/E690D oxidation was only detected at 1 mM H_2O_2 (Fig. 2*A*).

To test whether the Tyr(P) loop and the WPD motif were responsible for low oxidizability of RPTPα-D1, Tyr^{262} was mutated to valine, and Asp^{401} was mutated to glutamate. RPTPα-D1-Y262V and RPTPα-D1-D401E were much more sensitive to oxidation than wild type RPTPα-D1, in that appreciable oxidation was already detected at 31–62 μM H_2O_2 (Fig. 2*B*), similar to wild type RPTPα-D2. These results support the hypothesis that the Tyr(P) loop and WPD loop are important for susceptibility to oxidation of RPTPα-D1 and RPTPα-D2.

We determined the catalytic activity of RPTPα-D1, RPTPα-D2, and their mutants, allowing direct comparison of PTP activity and oxidizability. Consistent with previous results (12, 13, 33), the single point mutations in RPTPα-D2 enhanced its

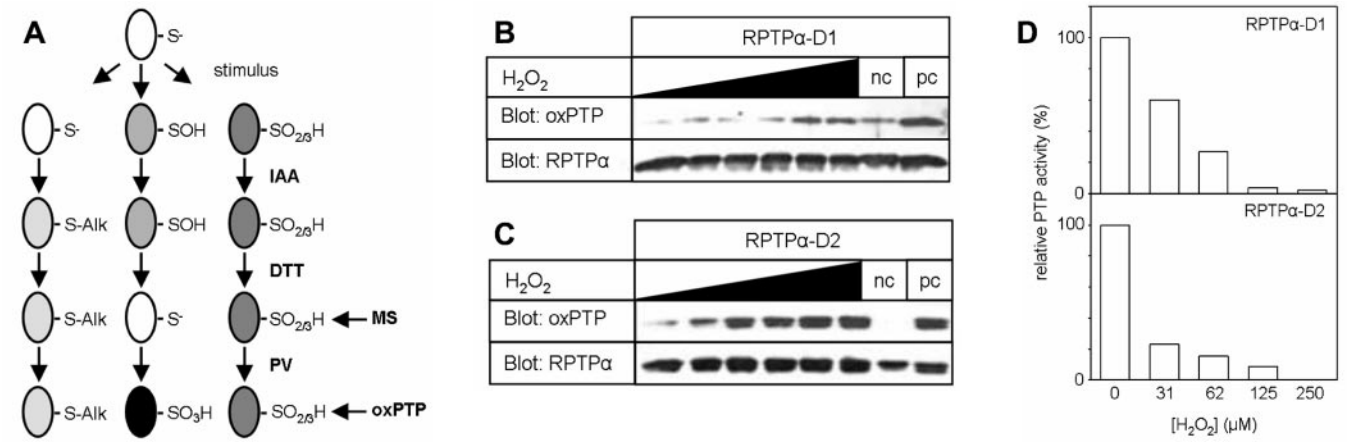

FIG. 1. **Differential oxidation of RPTPα-D1 and RPTPα-D2.** *A*, reduced (thiolate anion form, S^-), reversibly oxidized (sulfenic acid or sulfenamide; *SOH*), and irreversibly oxidized (sulfinic and sulfonic acid; $SO_{2/3}H$) PTP catalytic cysteines were detected as described in detail under "Materials and Methods" by subsequent alkylation with iodoacetic acid or iodoacetamide, reduction with DTT and detection by MALDI-ToF mass spectrometry (*MS*) or peroxidation with pervanadate (*PV*), and detection with oxPTP antibody. *B* and *C*, RPTPα-D1 (*B*) and RPTPα-D2 (*C*) were treated with increasing concentrations of H_2O_2 (31, 62, 125, 250, 500, and 1000 μM) and processed for detection of reversible oxidation, using the oxPTP antibody. The negative control (*nc*) was treated with DTT for 20 min. The positive control (*pc*) was not alkylated with iodoacetic acid, resulting in complete peroxidation in response to pervanadate. The samples were run on SDS-PAGE gels and blotted, and the immunoblots were probed with oxPTP antibody (*upper panels*). Equal amounts of fusion protein were loaded per lane, which was monitored by Coomassie Blue staining (data not shown) and by reprobing of the blots with anti-RPTPα antibodies (*lower panels*). Representative immunoblots, developed with ECL, are depicted here. *D*, GST fusion proteins encoding RPTPα-D1 (*top panel*) and RPTPα-D2 (*bottom panel*) were treated with different concentrations of H_2O_2 ranging from 31 to 250 μM. PTP activity was assayed using *p*-nitrophenylphosphate as a substrate and is depicted as a percentage of the maximal activity.

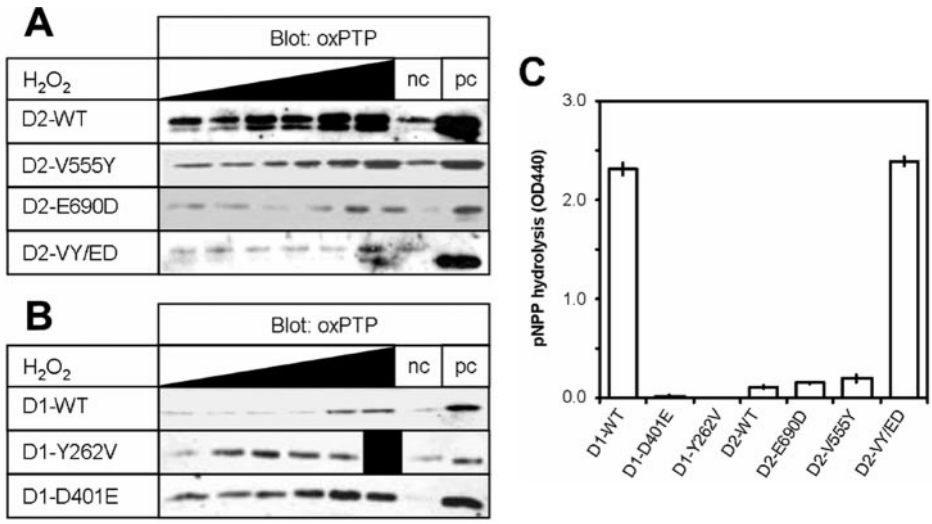

FIG. 2. **The Tyr(P) loop and the WPD loop are important for oxidizability.** *A* and *B*, RPTPα-D2 and mutants, V555Y, E690D, and V555Y/E690D (*VY/ED*) (*A*) and RPTPα-D1 and mutants, Y262V and D401E (*B*), were treated with increasing concentrations of H_2O_2 (31–1000 μM). Negative and positive controls were included for all mutants, and oxPTP immunoprobing was performed as described in the legend to Fig. 1. *C*, PTP activity of RPTPα-D1, RPTPα-D2, and their mutants was assayed using *p*-nitrophenylphosphate and quantified spectrophotometrically. The experiments were done in triplicate, and the standard deviations are indicated.

activity to some extent, whereas RPTPα-D2-V555Y/E690D exhibited catalytic activity similar to RPTPα-D1 (Fig. 2*C*). Mutation of Asp^{401} and Tyr^{262} completely abolished RPTPα-D1 activity (Fig. 2*C*), demonstrating that the Tyr(P) loop and WPD loop are essential for catalytic activity.

Mass spectrometry experiments were performed to further investigate oxidation of RPTPα-D1 and RPTPα-D2. Unfortunately, the Cys^{433} containing peptide of RPTPα-D1 did not resolve well in the MALDI-ToF spectra, and detection of Cys^{433} oxidation failed. The peptide containing Cys^{723} of RPTPα-D2 and its oxidized derivatives were readily detectable by MALDI-ToF (Fig. 3). At 1 mM H_2O_2, four peaks were evident in the spectra. As a result of the alkylation/reduction procedure, the reversibly oxidized Cys^{723} peptide was detected in its reduced form (*m/z* 2075.98), whereas alkylated Cys^{723} peptide corresponds to reduced Cys^{723} peptide (*m/z* 2133.00). The other two peaks, *m/z* 2107.98 and 2123.98, represent irreversibly oxidized RPTPα-D2, with relative mass shifts of +32 and +48 corresponding to the formation of the sulfinic and sulfonic acid species, respectively. Reversibly oxidized Cys^{723} peptide was already detected at the lowest concentrations of H_2O_2 (31 μM), and the fraction of reversibly oxidized Cys^{723} peptide increased gradually with increasing H_2O_2 concentrations (Fig. 3). The sulfinic acid species were only detected at high H_2O_2 concentrations (250 μM H_2O_2 and higher) and sulfonic acid only at 1 mM H_2O_2.

Based on the MALDI-ToF spectra, we calculated the fraction of reversibly oxidized Cys^{723} peptide, relative to the total amount of Cys^{723} peptide. Wild type RPTPα-D2 was highly sensitive to oxidation, whereas RPTPα-D2-E690D was moder-

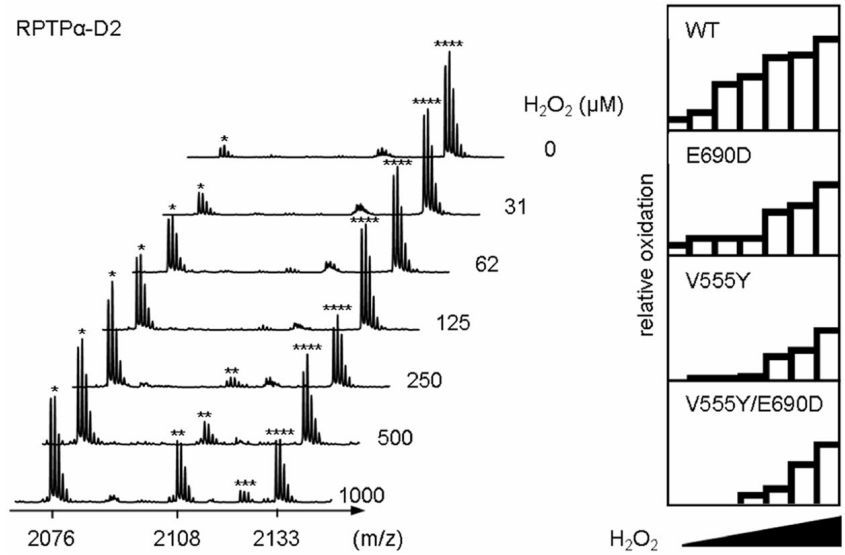

FIG. 3. **MALDI-ToF analysis of oxidation of RPTPα-D2 and mutants.** Purified RPTPα-D2 was treated with H_2O_2, and MALDI-ToF spectra were acquired as described under "Materials and Methods." The *m/z* range that contains the active site Cys^{723} peptide (residues 710–729) is depicted. Reversibly oxidized Cys^{723} peptide was detected in reduced form (*m/z* 2075.98, *), the sulfinic acid containing peptide shifted +32.00 to *m/z* 2107.98 (**), and the sulfonic acid containing peptide +48.00 to *m/z* 2123.98 (***). The reduced Cys^{723} containing peptide was detected in alkylated form with a shift in relative molecular mass of + 57.02 (*m/z* 2133.00, ****). Peak intensities of reduced and reversibly oxidized Cys^{723} peptides were determined from the MALDI-ToF spectra (*panels* on the *right*). Relative oxidation (*y* axes run from 0 to 100%) is plotted against increasing H_2O_2 concentrations (0, 31, 62, 125, 250, 500, and 1000 μM, *left* to *right*) for wild type (*WT*) and mutant RPTPα-D2 (as indicated).

ately sensitive, showing low levels of reversible oxidation at low H_2O_2 concentrations (Fig. 3). RPTPα-D2-V555Y and RPTPα-E690D/V555Y were the least sensitive to oxidation, because for both mutants significant oxidation was detected only at high concentrations of H_2O_2 (250 μM and higher; Fig. 3). The sulfinic and sulfonic acid form of Cys^{723} peptides in the MALDI-ToF spectra of RPTPα-D2 were not detected in any of the mutants (data not shown), again pointing toward a decreased sensitivity to oxidation for all mutants. The MALDI-ToF results are consistent with the oxPTP antibody data.

The oxidation and PTP activity results (Figs. 2 and 3) demonstrate that the two residues that determine the difference in catalytic activity between RPTPα-D1 and RPTPα-D2 are also responsible for the difference in oxidizability, which may suggest that PTP catalytic activity and oxidizability are linked.

Oxidizability of PTP1B and LAR—Our results suggest an inverse relation between catalytic activity and oxidizability. However, other PTPs that are catalytically active have been reported to be oxidized. Therefore, we investigated oxidation of PTP1B in parallel to RPTPα-D1 and RPTPα-D2. Oxidation of zebrafish PTP1B in which the oxPTP epitope (VHCSAG) is fully conserved (31) was readily detected at very low levels of H_2O_2 (31 μM), and PTP1B oxidation was maximal at 125–250 μM H_2O_2 (Fig. 4), similar to RPTPα-D2 (Fig. 1*C*). PTP1B is catalytically active (data not shown), arguing against an inverse correlation between catalytic activity and oxidation.

Next, we investigated oxidizability of the two catalytic domains of the RPTP, LAR. Both LAR-D1 and LAR-D2 were readily oxidized at low H_2O_2 concentrations (31 μM) with maximal oxidation levels at 125–250 μM, like RPTPα-D2 and PTP1B (Fig. 4). PTP activity assays that were done in parallel demonstrate that LAR-D1 is catalytically active and LAR-D2 is not (data not shown), consistent with previous reports (6). Taken together, the finding that the catalytically active PTP1B and LAR-D1 are readily oxidized indicates that there is not a strict inverse correlation between catalytic activity and oxidizability.

Because the Tyr(P) loop and WPD loop are conserved in PTP1B and LAR-D1, other features of the PTPs must play a role in determining the sensitivity to oxidation. Gly^{259} in PTP1B (Gln^{474} in RPTPα-D1 and His^{764} in RPTPα-D2) is an important determinant of substrate specificity (34) and determines the flexibility of Gln^{262} in the Q loop of the catalytic pocket (35). Gly^{259} might therefore be involved in the difference in oxidizability between PTP1B and RPTPα-D1. We mutated Gln^{474} in RPTPα-D1 and His^{764} in RPTPα-D2 to glycine, the

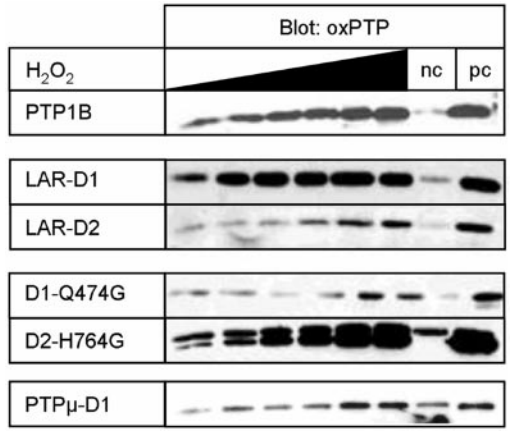

FIG. 4. **Differential oxidation of other PTPs.** PTP1B, LAR-D1, LAR-D2, RPTPα-D1-Q474G, RPTPα-D2-H764G, and PTPμ-D1 were treated with increasing concentrations of H_2O_2 (31–1000 μM), and reversible oxidation was detected as in Fig. 1. *nc*, negative control; *pc*, positive control.

residue at the corresponding position in PTP1B. RPTPα-D1-Q474G was poorly oxidized (Fig. 4), like wild type RPTPα-D1 (Fig. 1*B*). RPTPα-D2-H764G was readily oxidized (Fig. 4), like wild type RPTPα-D2 (Fig. 1*C*). Taken together, these results indicate that Gly^{259}, a determinant of substrate specificity in PTP1B and RPTPα, is not involved in differential oxidation of PTPs.

Extensive comparison of the primary sequences of the PTPs involved did not lead to the identification of residues that might be responsible for the difference in oxidizability, except for three residues that are conserved in RPTPα-D1 (^{331}TNL^{333}) and RPTPα-D2 (^{624}TEL^{626}) but not in PTP1B (^{109}NRV^{111}). Thr^{331} is buried in the catalytic pocket of RPTPα-D1 close to the catalytic Cys^{433} and therefore might have a role in oxidizability of Cys^{433}. We mutated Thr^{331} and the two following residues to the corresponding residues in PTP1B (T → N, TN → NR, and TNL → NRV) and investigated oxidizability and catalytic activity of the mutants. However, these mutants were not catalytically active, and oxidation was not detectable using the oxPTP antibody (data not shown), suggesting that these mutations severely disrupted the conformation of the catalytic pocket.

pH-dependent PTP Oxidation—Cysteine residues are most vulnerable to oxidation in the thiolate anion form. Therefore, the pH may be an important determinant for oxidation of PTPs.

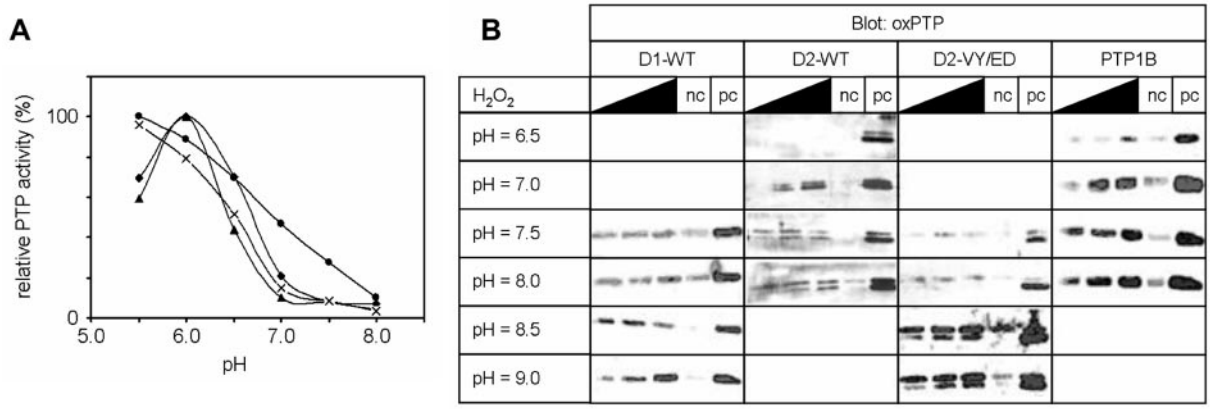

FIG. 5. **PTP oxidizability is pH-dependent.** *A*, PTP activity was assayed at pH 5.5–8.0 and is depicted relative to the maximal activity. ×, RPTPα-D1; ▲, RPTPα-D2; ♦, RPTPα-D2-V555Y/E690D; ●, PTP1B. *B*, PTPs were treated with increasing concentrations of H_2O_2 (31, 62, or 125 μM) at pH 6.5–9.0, and reversible oxidation was assessed as in Fig. 1. *nc*, negative control; *pc*, positive control.

It is well known that the catalytic activity of PTPs is strongly dependent on pH (36, 37). We assayed the PTP activity of RPTPα-D1, RPTPα-D2, RPTPα-D2-V555Y/E690D, and PTP1B at different pH. PTPs are highly active at low pH (optimum activity at pH 5.5–6.0 for all PTPs tested). PTP activity decreased rapidly with increasing pH to 10% of the maximum activity at pH 8.0. It is noteworthy that although the absolute activity of the four PTPs tested is different (PTP1B > RPTPα-D1 > RPTPα-D2-V555Y/E690D ≫ RPTPα-D2), the pH-dependent decrease in activity is very similar (Fig. 5*A*).

To investigate whether oxidation is a pH-dependent process, H_2O_2 treatment was done at a range of different pHs. Three H_2O_2 concentrations were used that are indicative of oxidizability, 31, 62, and 125 μM. Strikingly, the oxidizability of RPTPα-D1 was clearly dependent on pH in that RPTPα-D1 was oxidized in response to low levels of H_2O_2 (31 μM) at pH 8.5 and 9.0 (Fig. 5*B*). RPTPα-D2 oxidation was not affected by elevating the pH, but lowering the pH to 7.0 and 6.5 gradually reduced oxidation of RPTPα-D2 (Fig. 5*B*). PTP1B was sensitive to oxidation at pH 7.5 and, like RPTPα-D2, lost its sensitivity when the pH was lowered to 6.5. RPTPα-D2-V555Y/E690D responded to different pHs much like RPTPα-D1, in that RPTPα-D2-V555Y/E690D was oxidized at high pH (pH 8.5 and 9.0). Taken together, these results demonstrate that oxidation of PTPs is strongly dependent on the pH, suggesting that the microenvironment of the catalytic cysteines has an important role.

PTP loop Arginine Is Important for Oxidizability—The crystal structures of the PTPs involved have all been solved: RPTPα-D1 (38), RPTPα-D2 (11), PTP1B (9), and LAR-D1 and LAR-D2 (10). Comparison of the microenvironment of the catalytic cysteine of these five PTPs indicated that the absolutely conserved PTP loop arginine is oriented differently in RPTPα-D1 than in RPTPα-D2, PTP1B, or LAR (Fig. 6). We determined the distance between the Sγ atom of the catalytic cysteine and the three N atoms of the guanidinium group of the arginine (Table I). Strikingly, the Nη_1 atom was much closer to the Sγ atom of the catalytic cysteine in RPTPα-D1 than in the other PTPs (4.9 Å *versus* 7.1–8.0 Å; Table I). The Nϵ atoms were located at similar distances in all five PTPs (5.2–6.1 Å), whereas the Nη_2 atoms were partially buried in most structures at similar distances to the Sγ atom (6.1–7.3 Å). The proximity of the Nη_1 atom to the Sγ atom may account for the difference in oxidizability between RPTPα-D1 and other PTPs, because the guanidinium group of Arg^{439} may sterically hinder the reaction of H_2O_2 with the thiolate group of Cys^{433}.

We analyzed the crystal structures of other classical PTPs, RPTPμ-D1, TC-PTP, Shp-1, Shp-2, PTP-SL, and YOP51. We found that only in RPTPμ-D1, was the guanidinium group of the PTP loop arginine oriented similarly as in RPTPα-D1 (Fig. 6), and the Nη_1 atom was relatively close to the Sγ atom (5.5 Å; Table I). Indeed, RPTPμ-D1 was only oxidized at high H_2O_2 concentrations (Fig. 4), similar to RPTPα-D1. Our results are consistent with a crucial role for the PTP loop arginine in the oxidizability of PTPs.

DISCUSSION

Oxidation is an attractive regulatory mechanism for PTPs, because ROS are readily formed in response to various stimuli, and oxidation is reversible. Here, we report that PTPs were oxidized differentially, which may be important for fine-tuning of the cellular responses to stimuli. We demonstrate that oxidizability and catalytic activity of RPTPα-D1 and RPTPα-D2 were reversed upon mutation of two residues in the Tyr(P) loop and WPD loop that are absolutely conserved in catalytically active PTPs but not in RPTP-D2s. However, these two residues cannot explain the difference in oxidizability between RPTPα-D1 on the one hand and the catalytically active PTPs, PTP1B, and LAR-D1 on the other, because these residues are conserved in all active PTPs. We found that oxidation of PTPs was highly dependent on pH, and we identified the PTP loop arginine as an important determinant in oxidizability.

oxPTP immunoprobing, PTP activity assays, and MALDI-ToF mass spectrometry consistently showed differential oxidation of purified RPTPα-D1 and RPTPα-D2 *in vitro*. We have previously shown that Cys^{723}, the catalytic cysteine of RPTPα-D2, is preferentially oxidized in living cells in response to UV treatment (28). Mutation of Cys^{723}, but not Cys^{433}, abolished oxidation of full-length RPTPα in living cells. Moreover, differential oxidizability of RPTPα-D1 and RPTPα-D2 is consistent with previous data that RPTPα-D2 rather than RPTPα-D1 is the redox sensor. Mutation of Cys^{723} renders full-length RPTPα at least in part insensitive to oxidation, in that H_2O_2 treatment or UV treatment of living cells completely inactivates wild type RPTPα, but not RPTPα-C723S (8, 28). H_2O_2-induced inactivation of RPTPα is due to a conformational change in RPTPα-D2, which leads to stabilization of an inactive RPTPα dimer conformation (8, 29). How RPTPα-D2 changes conformation in response to H_2O_2 remains elusive and will require elucidation of the crystal structure of oxidized RPTPα-D2. The catalytic Cys^{723} is required for the conformational change (8), and recently we found evidence that none of the other cysteines in RPTPα is involved in H_2O_2-induced stable dimer formation (39), ruling out the possibility of intramolecular disulfide bond formation, which has been observed in nonclassical Cys-based PTPs, including LMW-PTP (40), PTEN (41), and CDC25 (42). It is likely that the conformational

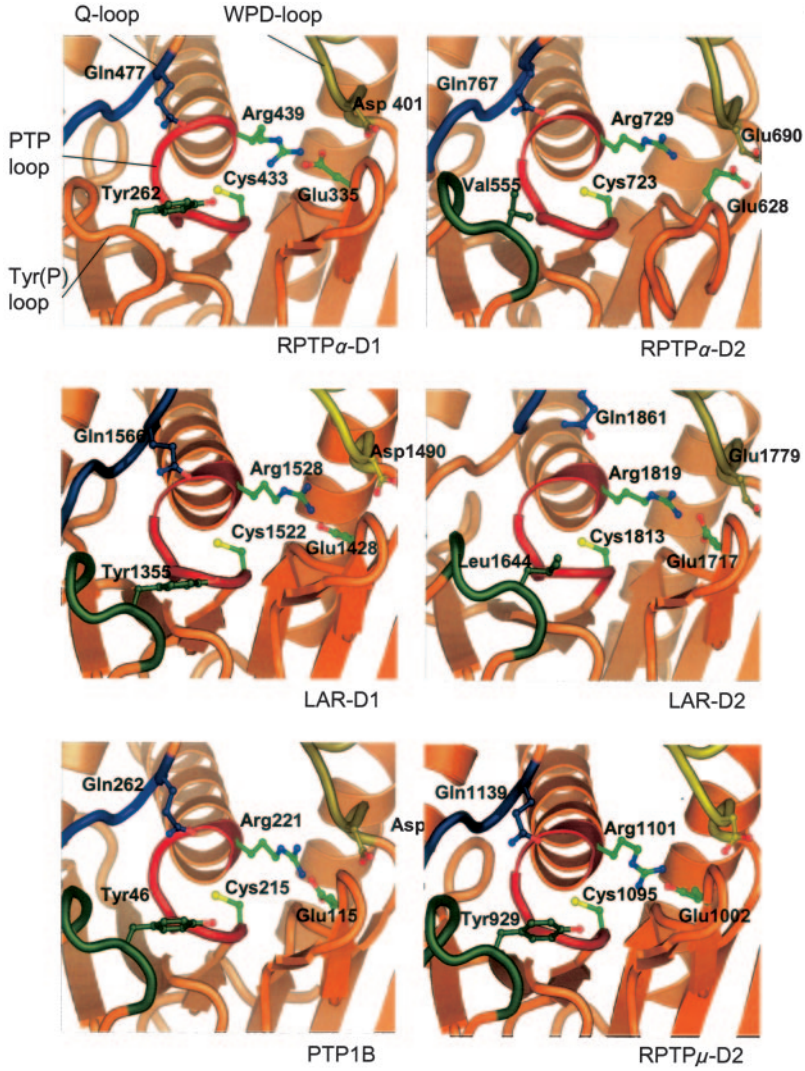

FIG. 6. **Structural basis for differential oxidation of PTPs.** Shown is a comparison of the microenvironment of the catalytic cysteine in RPTPα-D1, RPTPα-D2, LAR-D1, LAR-D2, PTP1B, and RPTPμ-D1. The PTP loop is in *red*, the Tyr(P) loop is in *green*, the Q loop is in blue, and the WPD loop is in *olive green*. Note the difference in orientation of the guanidinium group of Arg^{439} in RPTPα-D1 and Arg^{1101} in RPTPμ-D1, compared with the PTP loop arginine in the other PTPs.

TABLE I
Difference in microenvironment of the catalytic cysteine in RPTPα -D1 and RPTPμ -D1 compared to other PTPs

The distances between the catalytic cysteine Sγ atom and the PTP loop arginine guanidinium group N atoms (Å) were determined.

PTP	Nε-Sγ	$N\eta_1$-Sγ	$N\eta_2$-Sγ
RPTPα-D1	5.593	4.857	6.904
RPTPα-D2	5.724	7.463	7.041
LAR-D1	6.043	7.992	7.127
LAR-D2	6.113	7.951	7.329
PTP1B	5.232	7.102	6.081
RPTPμ-D1	4.934	5.465	6.874
TC-PTP	5.066	7.007	7.304
Shp-1	5.153	6.256	7.058
Shp-2	5.701	7.418	7.223
PTP-SL	6.088	7.894	6.906
YOP51	5.818	7.504	6.073

change in RPTPα-D2 is initiated by cyclic sulfenamide formation, which leads to extensive reorganization of the catalytic site of PTP1B (19, 20).

Although oxidizability of RPTPα-D1 and RPTPα-D2 was reversed upon mutation of only two residues in the Tyr(P) loop and WPD loop (Figs. 2 and 3), this cannot explain the difference in oxidizability between PTP1B, LAR-D1, and RPTPα-D1 (Fig. 4), because the Tyr(P) loop and WPD loop are conserved in all three active PTPs. Oxidation of PTPs is strongly dependent on pH (Fig. 5), suggesting that the microenvironment of the catalytic cysteine is an important determinant for oxidizability. Comparison of the crystal structures of the classical PTPs suggested that the orientation of the PTP loop arginine was important. The orientation of the PTP loop arginine in RPTPμ-D1 suggested that RPTPμ-D1 would not be highly susceptible to oxidation. Indeed, like RPTPα-D1, RPTPμ-D1 was relatively insensitive to oxidation (Fig. 4). The proximity of the PTP loop arginine to the catalytic cysteine may sterically hinder the reaction of H_2O_2 with the thiolate anion group. To directly test the role of the PTP loop arginine in oxidizability, we mutated Arg^{439} to lysine in RPTPα-D1. Unfortunately, GST-RPTPα-D1-R439K was not stable, and we did not obtain sufficient material to determine the oxidation of this fusion protein.

It is not clear why the guanidinium group of Arg^{439} is oriented differently in RPTPα-D1 than in most other PTPs. Unlike the other crystal structures, RPTPα-D1 formed dimers in the crystals in which a helix loop-helix wedge-like structure inserts into the catalytic site of the dyad-related monomer (38), which may affect the orientation of the guanidinium group. However, the position of the PTP loop arginine and particularly its guanidinium group in RPTPμ-D1, which did not form dimers like RPTPα-D1 in crystals (43), is similar to that in RPTPα-D1, indicating that this conformation is not driven by dimerization.

It is noteworthy that differential oxidation of PTPs is especially obvious at relatively low H_2O_2 concentrations. Cellular

stimuli induce H_2O_2 concentrations in a range, similar to the H_2O_2 concentrations we have used for our experiments. For instance, platelet-derived growth factor induces ROS levels in cells that are comparable with the levels in cells resulting from exogenously added H_2O_2 concentrations of 0.1–1.0 mM (21). Differential oxidation of PTPs is important for fine-tuning of the cellular response to ROS production. Apparently, different inactivating mechanisms have evolved in the RPTPs: direct oxidation of the catalytic cysteine in LAR and indirect inactivation through oxidation of the catalytic cysteine in RPTPα-D2, resulting in stabilization of an inactive dimer conformation (8, 29). LAR-D2 does undergo a conformational change in response to H_2O_2, like RPTPα-D2, and H_2O_2 induces heterodimer formation between full-length RPTPα and LAR (44). Whether oxidation of LAR-D2 is required for full inactivation of LAR remains to be determined.

In conclusion, we demonstrate that PTPs are differentially oxidized, and we provide evidence for the underlying mechanism. It will be interesting to see whether other PTP family members, including the nonclassical Cys-based PTPs, are differentially oxidized as well.

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Differential Oxidation of Protein-tyrosine Phosphatases

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