H$_2$O$_2$ induced intermolecular disulfide bond formation between
Receptor Protein-Tyrosine Phosphatases

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Running title: Intermolecular disulfide bond formation between RPTPs
Abstract

Receptor Protein-Tyrosine Phosphatase α belongs to the subfamily of receptor-like protein-tyrosine phosphatases that are characterized by two catalytic domains of which only the membrane-proximal one (D1) exhibits appreciable catalytic activity. The C-terminal catalytic domain (D2) regulates RPTPα catalytic activity by controlling rotational coupling within RPTPα dimers. RPTPα-D2 changes conformation and thereby rotational coupling within RPTPα dimers in response to changes in the cellular redox state. Here we report a decrease in motility of RPTPα from cells treated with H₂O₂ on non-reducing SDS-PAGE gels to a position that corresponds to RPTPα dimers, indicating intermolecular disulfide bond formation. Using mutants of all individual cysteines in RPTPα and constructs encoding the individual PTP domains, we located the intermolecular disulfide bond to the catalytic Cys723 in D2. Disulfide bond formation and dimer stabilization showed similar concentration- and time-dependencies. However, treatment of lysates with dithiotreitol abolished intermolecular disulfide bonds, but not stable dimer formation. Intermolecular disulfide bond formation and rotational coupling were also found using a chimera of the extracellular domain of RPTPα fused to the transmembrane and intracellular domain of LAR. These results suggest that H₂O₂ treatment leads to oxidation of the catalytic Cys in D2, which then rapidly forms a disulfide bond with the D2 catalytic Cys of the dyad-related monomer, rendering an inactive RPTP dimer. Recovery from oxidative stress first leads to the reduction of the disulfide bond followed by a slower refolding of the protein to the active conformation.
Introduction

Protein-tyrosine phosphatases (PTPs) form a family of enzymes that catalyse the dephosphorylation of tyrosine residues in proteins. They are characterized by one or two catalytic domains containing a signature sequence (I/V)HCXAGXXR(S/T/G) including a catalytic cysteine (reviewed in Refs. 1 and 2). This cysteine forms a thiol-phosphate intermediate in the dephosphorylation reaction and is therefore essential for enzyme activity (3). Because of the low pKa of the catalytic cysteine, PTPs are very susceptible to oxidation (reviewed in Ref. 4). Reactive oxygen species (ROS) induce oxidation of catalytic cysteines, thereby inactivating these PTPs (5-8). Extracellular stimuli like growth factors and UV result in an increase in intracellular ROS and oxidation of PTPs (5, 9-12). Inhibition of enzyme activity by oxidative stress is increasingly recognized as an important mechanism of regulation of the PTP family. Therefore, PTPs may serve as sensors of the cellular redox state.

RPTPα belongs to the receptor like PTPs that are characterized by a single transmembrane domain. RPTPα has two catalytic domains of which the N-terminal one (D1) contains almost all of the catalytic activity of the enzyme. RPTPα was found to constitutively form dimers in the cell membrane (13, 14) and activity of the dimer is dependent on the relative orientation of the two monomers in the dimer (15). Studies on the crystal structure of RPTPα-D1 indicate that a helix-loop-helix wedge-like structure to the N-terminal side of D1 occludes the catalytic site of the dyad-related monomer (16). The RPTP CD45 is also regulated by dimerization (17, 18). Mutations in the wedge-like structure of CD45 and RPTPα abolished dimerization.
induced inactivation (15, 19) proving that the wedge-like structure is essential for the regulation of RPTP activity by rotational coupling of the monomers in the dimer.

Previously, we showed that the catalytically inactive C-terminal PTP domain (D2) of RPTPs has a regulatory role (20, 21). Recent studies on oxidative stress and RPTPα indicate that D2 acts as a redox sensor (22). Using an antibody that recognizes oxidized classical PTPs, RPTPα-D2 exhibits a higher susceptibility to oxidation than RPTPα-D1 (12). Furthermore, H$_2$O$_2$ treatment induces a rapid, reversible and catalytic site Cys723-dependent in vivo change in protein conformation, rendering a more stable, catalytically inactive RPTPα dimer (22).

LMW-PTP, CDC25 and PTEN, non-classical members of the PTP family form intramolecular disulfide bonds between the catalytic cysteine and an adjacent cysteine upon oxidation, which protects the catalytic cysteine against irreversible further oxidation (7, 8, 23). Using RPTPα, we investigated whether disulfide bond formation is involved in oxidative stress induced conformational changes in classical PTPs. Here we report fast and reversible in vivo intermolecular disulfide bond formation upon H$_2$O$_2$ treatment. By alternatively mutating all cysteines in RPTPα to serines, we localized the intermolecular disulfide bond between the cysteines at position 723 of the two monomers in the dimer. Studies with a chimeric protein encoding the RPTPα ectodomain fused to the LAR transmembrane and intracellular domains indicate that intermolecular disulfide bond formation and a subsequent change in dimer conformation, is not restricted to RPTPα but is a general theme for RPTPs with two intracellular PTP domains. Oxidation-induced stable dimer formation persisted longer than the disulfide bonds and reduction of the disulfide bonds did not disrupt stable dimer formation, which is consistent with a model in which reversible oxidation initiates, but does not maintain stable dimer formation.
Materials and Methods

Constructs

SV40-driven expression vectors have been described for full length HA-
RPTPα (24), Myc-RPTPα (22), HA-RPTPα-C433S, HA-RPTPα-C723S and HA-
RPTPα-C433S/C723S (25). Individual Cys→Ser mutants were generated by PCR
using appropriate oligonucleotides and were verified by sequencing. Expression
vectors for the individual RPTPαD1 and RPTPαD2 domains were described
previously (20, 22). The fusion protein encoding the RPTPα ectodomain, human
LAR transmembrane and intracellular domain fusion protein (EDα-LAR) was
generated by PCR. The ectodomain of HA-tagged RPTPα (residues 1 – 141) was
amplified by PCR and a KpnI-site was added to the 5’side. The transmembrane and
cytoplasmic domains of human LAR (residues 1235 – stop) were amplified by PCR
with a KpnI site engineered on the 3’ end. These two PCR products were cloned into
the SV40 promoter-driven expression vector pSG5 and the entire construct was
verified by sequencing.

Cell culture and transfection

Cos-1 cells were routinely grown in DMEM/F12 supplemented with 7.5% fetal
bovine serum. Transient transfection of Cos-1 cells was done by calcium phosphate
precipitation as described previously (24). The medium was refreshed the day after
transfection using medium without serum and experiments were performed after
another 16 h. Before cell lysis, cells were stimulated with H2O2 (Sigma-Aldrich)
and/or pervanadate as indicated in medium without serum.
Stable dimer detection by co-immunoprecipitation and non-reducing SDS-page

After stimulation, cells transfected with both Myc-tagged RPTPα and HA-tagged RPTPα constructs were lysed in cell lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1 mM MgCl₂, 10% glycerol 1% TX-100, aprotinin and leupeptin) in the presence of 20 mM iodoacetamide when indicated and centrifuged at 14000 g for 15 min. To check for protein expression and to study homodimer formation by intermolecular disulfide bond formation, samples from the supernatant were diluted 1:1 in 2x Laemmli sample buffer with β-mercaptoethanol (reducing conditions), or without (non-reducing conditions), as indicated. Subsequently, the samples were boiled for 5 min and loaded onto 5% SDS-PAGE gels. The rest of the supernatant was rotated for 2 h at 4°C with anti-HA antibody linked to protein A-Sepharose beads. The beads were spun down and washed four times with HNTG (20 mM HEPES pH 7.4, 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol). The samples were boiled in Laemmli sample buffer and separated on 7.5% SDS-PAGE gels. The material on the gel was transferred to PVDF membrane by semi-dry blotting. The membranes were stained with Coomassie Blue, blocked for 1 h at room temperature in TBST (50 mM Tris, pH 8.0; 150 mM NaCl, 0.05% Tween-20) containing 5% milk and incubated for another hour with anti-HA, anti-Myc or anti-RPTPα antibody 5478 (26) in TBST + 5% milk. The blots were washed 3 times with TBST and incubated with HRP-conjugated secondary antibody for 1 h. After washing with TBST the immunoreactivity on the membranes was visualized using enhanced chemiluminescence (ECL) according to standard protocols.
HA-accessibility assay

The conformation of the extracellular domain was detected by the accessibility of the HA-tag at the N-terminal side of the extracellular domain of HA-α and of the EDα-LAR chimeric protein. The procedure is described in detail in (27). In short, after stimulation, the living cells were incubated ice-cold with anti-HA-antibody for 1 h. After extensive washing, cells were lysed and the antibody-bound fraction of the protein was pulled down from the lysate with protein A-sepharose. This part of the total amount of transfected protein is called the accessible fraction. The rest of the HA-tagged protein, the non-accessible fraction, was immunoprecipitated from the remaining lysate using protein A-sepharose bound anti-HA-antibody. The immunoprecipitating proteins were visualized following SDS-PAGE by immunoblotting as described above.
Results

Rapid formation of intermolecular S-S bridges between RPTPα monomers in response to H$_2$O$_2$

Previously, we showed that H$_2$O$_2$ treatment of cells transfected with differentially tagged RPTPα constructs leads to the formation of stable RPTPα dimers as detected by co-immunoprecipitation of the two constructs (22). To study whether dimer-stabilization coincides with the formation of intermolecular disulfide bond(s) we co-transfected Cos-1 cells with both HA- and Myc-tagged RPTPα. Cells were stimulated with different concentrations of H$_2$O$_2$ for 5 min (Fig. 1A). The cells were lysed, and samples were run on SDS-PAGE gels under reducing and non-reducing conditions. HA-RPTPα was immunoprecipitated from the remaining lysates and co-immunoprecipitation of Myc-tagged RPTPα was detected. H$_2$O$_2$ treatment dose-dependently induced RPTPα to migrate at a position that corresponds to the double molecular weight of an RPTPα monomer on SDS-PAGE gels under non-reducing conditions (Fig. 1A, upper panel). The RPTPα bands that appeared on SDS-PAGE gels under non-reducing conditions after H$_2$O$_2$ stimulation were absent under reducing conditions (data not shown, cf. Fig. 5B). As shown in the middle panel of Fig. 1A, the increasing amount of RPTPα dimers under non-reducing SDS-PAGE conditions coincided with the co-immunoprecipitation of Myc-tagged RPTPα with HA-RPTPα.

Figure 1B shows that both dimer-formation detected by SDS-PAGE under non-reducing conditions and by co-immunoprecipitation of differentially tagged RPTPα constructs coincided in time. Dimers were already prominently and maximally formed after 1 min of H$_2$O$_2$ treatment and declined slightly after
approximately 5 min. These results show that intermolecular disulfide bonds are formed between RPTPα monomers with similar kinetics as stable co-immunoprecipitating RPTPα dimers.

*Intermolecular S-S bridges between catalytic cysteines of RPTPα*

To map the cysteines involved in H$_2$O$_2$ induced intermolecular disulfide bond formation, we mutated all cysteines individually in the HA-tagged RPTPα construct to serines. Cells, transfected with Myc-tagged wild-type RPTPα together with wild-type HA-tagged RPTPα or one of the HA-tagged RPTPα cysteine to serine mutants, were stimulated with H$_2$O$_2$ or left untreated. Cells were lysed and samples were taken to run on SDS-PAGE gels for detection of expression levels and intermolecular disulfide bond formation (non-reducing conditions). HA-tagged RPTPα was precipitated from the rest of the lysates and co-immunoprecipitation of myc-tagged RPTPα was detected. As shown in Figure 2A, Myc-RPTPα co-precipitated with all but one cysteine to serine mutant; only Cys723 was found to be essential for the H$_2$O$_2$ induced stable dimer formation as detected by co-precipitation of differentially tagged RPTPα monomers. Although some of the other cysteine to serine mutants were poorly expressed (e.g. C339S, C421S) they still showed appreciable co-immunoprecipitation with Myc-RPTPα. Note that the RPTPα-C734S is lacking because expression was undetectable. Figure 2B demonstrates that only the C433S and C723S mutant showed a decreased amount of intermolecular disulfide bond formation upon peroxide treatment, while this was unaffected in the rest of the cysteine mutants.

To investigate the role of the catalytic cysteines in intermolecular disulfide bond formation and stable dimerization in more detail, we transfected cells with Myc-
tagged wild type RPTPα together with either HA-tagged wildtype RPTPα, HA-tagged RPTPαC433S, HA-tagged RPTPαC723S or the double mutant HA-tagged RPTPαC433S/C723S. The C433S mutant behaved like wildtype with respect to H₂O₂ induced co-immunoprecipitation of Myc-tagged RPTPα and intermolecular disulfide bond formation (Fig. 3). The C723S mutant did not show any co-immunoprecipitation with Myc-tagged RPTPα and did not show a band migrating at the molecular level of RPTPα dimers on non-reducing SDS-PAGE gels. However, a band that migrated slower than the expected dimeric RPTPα band was still present in the C723S mutant after H₂O₂ treatment (Fig. 3). Interestingly, this band was strongly reduced in cells transfected with HA-RPTPαC433S or HA-RPTPαC433S/C723S in response to H₂O₂, suggesting that the catalytic cysteine of the RPTPα-D1 is involved in the formation of this slower migrating complex.

Using constucts encoding the individual catalytic domains D1 and D2 respectively, we found that only D2 forms intermolecular disulfide bonded dimers that migrate in SDS-PAGE gels under non-reducing conditions with an apparent molecular weight of D2-D2 homodimers, which was completely dependent on Cys723 (Fig. 4). D1 did not form intermolecular disulfide bonds upon treatment of cells with 1 mM H₂O₂ (Fig. 4). Since full length RPTPαC734S was not expressed in cells, we assessed involvement of Cys734 in intermolecular disulfide bond formation in the D2 domain by itself. Importantly, D2-C734S acted in a similar fashion as wild type D2 indicating that Cys734 is not involved in intermolecular disulfide bond formation. These results suggest that upon H₂O₂ treatment, the catalytic Cys723 forms a disulfide bond, cross-linking RPTPαD2s.

To check whether intermolecular disulfide bond formation upon oxidative stress is a general theme of RPTPs containing two PTP domains, a chimeric
construct was made in which the HA-tagged extracellular domain of RPTPα was fused to the transmembrane- and intracellular domain of LAR (HA-EDα-LAR). It has been shown that LAR-D2, like RPTPα-D2, shows a conformational change upon oxidative stress (21). Like HA-RPTPα (27), HA-EDα-LAR shows a change in rotational coupling of the dimer upon H2O2 treatment as detected by the accessibility of the HA-tag on living cells transfected with HA-EDα-LAR (Fig. 5A). As shown in Figure 5B, H2O2 induced HA-EDα-LAR to migrate at a level that corresponds to the double molecular weight of an HA-EDα-LAR dimer on SDS-PAGE gels under non-reducing conditions, indicating that oxidation-induced intermolecular disulfide bond formation is a phenomenon not limited to RPTPα.

Role of intermolecular disulfide bonds in stable RPTPα dimer formation

Since Cys723 is required both for intermolecular disulfide bond formation and for stable dimer formation as detected by co-immunoprecipitation of differentially tagged RPTPα monomers, and both emerge with the same kinetics in response to H2O2 treatment (Fig. 1B), we investigated whether intermolecular disulfide bond formation is responsible for co-immunoprecipitation of differentially tagged RPTPα monomers. Analysis of the time-course of recovery of H2O2 induced RPTPα dimer formation indicated a difference in persistence of the intermolecular disulfide bonds and RPTPα dimer stabilization. Cells transfected with wildtype RPTPα were treated for 5 min with 1 mM H2O2. The medium was replaced with medium without H2O2 and the cells were left to recover for 0 - 2 h. Figure 6 shows that intermolecular disulfide bonds were less persistent than co-immunoprecipitating RPTPα dimers; while high molecular weight bands on SDS-PAGE gels under non-reducing conditions
disappeared after 90 min of recovery, still an appreciable amount of Myc-tagged RPTPα co-immunoprecipitated with HA-tagged RPTPα after 2 h of recovery.

To investigate whether stable RPTPα dimers can indeed persist without an intermolecular disulfide bond, we treated cells with H2O2 and lysed the cells in the presence or absence of 1 mM dithiotreitol (DTT) (Fig. 7). We found that reduction with DTT abolished the intermolecular disulfide bonds, while stabilized RPTPα dimers were still detected. Pervanadate is a strong oxidizing agent that triply oxidizes the PTP catalytic cysteine into the sulfonic acid form, which cannot form disulfide bonds (28). As shown in Figure 7, treatment of cells with pervanadate did not induce stable dimer formation nor intermolecular disulfide bond formation. To confirm triple oxidation upon pervanadate treatment, we used an antibody generated against triply oxidized PTP-catalytic cysteine, anti-oxPTP (12). As shown in Figure 8, 1 mM pervanadate indeed led to the formation of triply oxidized RPTPα, while no triply oxidized RPTPα is formed after 5 min treatment with 1 mM H2O2. Interestingly, when cells are first treated with H2O2 for 5 min and subsequently with 1 mM pervanadate for 5 min, stable dimers and intermolecular disulfide bonds are formed comparable to treatment with H2O2 alone, indicating that disulfide bond formation protects against irreversible oxidation. Conversely, when cells are first stimulated with pervanadate and then with H2O2, a significantly smaller, but appreciable amount of stable dimers and intermolecular disulfide bonds are formed, indicating that oxidation by pervanadate is not complete. Taken together, these results strongly suggest that reversible oxidation leading to the formation of intermolecular disulfide bonds initiates stable dimer formation, but is not required for the persistence of stable dimers.
Discussion

The conserved cysteines in the catalytic pocket of PTPs are very sensitive to oxidation (5-8). Oxidation of the cysteine to sulenic acid leads to reversible inactivation of the enzyme while further oxidation to sulfinic and sulfonic acid irreversibly inactivates the enzyme. Recently, PTP1B was revealed to form cyclic sulenyl-amide after oxidation by H$_2$O$_2$, in which the sulfur of the catalytic cysteine is covalently linked to the main chain nitrogen of the adjacent serine residue (29, 30). Formation of this cyclic sulenyl-amide results in a rapid elimination of oxygen from sulenic acid and suppresses further, irreversible oxidation. In this paper we show that RPTP$_{\alpha}$ forms intermolecular disulfide bonds between the catalytic cysteines of the membrane distal PTP domain upon treatment of cells with H$_2$O$_2$.

Recently, by using a conformation-sensitive antibody method, we found that H$_2$O$_2$ treatment leads to a change in rotational coupling of the RPTP$_{\alpha}$ dimers, which is dependent on the catalytic Cys723 of the membrane distal PTP domain (27). The conformation of untreated RPTP$_{\alpha}$ dimers is similar to the conformation of the active constitutive dimer mutant RPTP$_{\alpha}$-F135C, while the H$_2$O$_2$ treated RPTP$_{\alpha}$ dimers adopt the conformation as found with an inactive constitutive dimer mutant, RPTP$_{\alpha}$-P137C. The H$_2$O$_2$-induced intermolecular disulfide bond formation, like stabilized dimer formation and rotational coupling of RPTP$_{\alpha}$ dimers, all depend on the catalytic cysteine of the membrane distal PTP domain. Furthermore, intermolecular disulfide bond formation and stable dimer formation are formed with similar kinetics upon treatment of cells with H$_2$O$_2$. These results suggest that intermolecular disulfide bond formation between the Cys723s of the RPTP$_{\alpha}$ monomers in the dimer induces rotational coupling and stable dimer formation. However, we cannot exclude the
possibility that oxidation to cyclic sulfenyl-amide, without disulfide bond formation, by itself can induce rotational coupling and thereby stable dimer formation. Previously, H$_2$O$_2$-induced conformational changes of RPTP$\alpha$-D2 were studied using the fluorescence resonance energy transfer technique (FRET; 22). A decrease in FRET was found upon H$_2$O$_2$-treatment of lysates containing the CFP-RPTP$\alpha$-D2-YFP construct. Interestingly, treatment of cell lysates with H$_2$O$_2$ did not lead to the formation of intermolecular disulfide bonds between RPTP$\alpha$ monomers (data not shown). This indicates that the monomers have to be in very close proximity of each other for disulfide bond formation, like in the cell membrane where they constitutively form dimers. Furthermore, these results rule out the possibility that the H$_2$O$_2$-induced conformational change in RPTP$\alpha$-D2 is caused by the formation of disulfide bonds between RPTP-D2s. It is likely that the reversible conformational change in RPTP$\alpha$-D2 is caused by the formation of cyclic sulfenyl-amide, which reportedly leads to conformational changes of the catalytic site (29, 30). Cyclic sulfenyl-amide formation leads to the opening up of the catalytic pocket, making it more shallow and rendering the catalytic cysteine more accessible for intermolecular disulfide bond formation with the thiolate anion Cys723 from the dyad-related RPTP$\alpha$ monomer.

In line with the cyclic sulfenyl-amide, intermolecular disulfide bond formation excludes oxygen from the structure and protects against irreversible oxidation as shown here by the inability of pervanadate to triply oxidize the catalytic cysteine after peroxide treatment. This mechanism of intermolecular disulfide bond formation between the catalytically inactive RPTP-D2s may protect membrane localized PTPs against irreversible oxidation by free radicals that are abundantly formed upon cell stimulation, such as growth factor receptor signaling.
From our recovery studies (Fig. 5), intermolecular disulfide bond formation seemed to reverse faster than stable dimer formation. Indeed, DTT treatment of lysates from cells stimulated with H$_2$O$_2$ immediately reduced the intermolecular disulfide bonds between RPTP$\alpha$ monomers, while differentially tagged monomers still co-immunoprecipitated. In line with this, recovery from the H$_2$O$_2$-induced conformational changes of RPTP$\alpha$-D2 in cells as detected by FRET was relatively slow (22), indicating that refolding of RPTP$\alpha$-D2 is independent of disulfide bond reduction but probably due to another intrinsic property of the protein.

Taken together, these findings led us to the model that H$_2$O$_2$-induced oxidation of the RPTP$\alpha$-Cys723 to cyclic sulfenyl-amide leads to a conformational change of RPTP$\alpha$-D2s and thereby a change in rotational coupling resulting in dimer stabilization. This cyclic-sulfenyl amide reacts rapidly with the thiolate anion Cys723 of the dyad-related monomer to form a disulfide bond. Recovery from oxidative stress first leads to a reduction of the intermolecular disulfide bond followed by a slower refolding of the protein. Formation of intermolecular disulfide bonds between RPTPs renders both monomers in the dimer in an inactive conformation and at the same time it protects against irreversible further oxidation.
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Footnotes: 1. The abbreviations used are:

DTT; dithiotreitol

ED; extracellular domain

FRET; fluorescence resonance energy transfer technique

HA; haemagglutinin

LAR; leukocyte common antigen-related

LMW-PTP; low molecular weight PTP

PTEN; phosphatase and tensin homolog

PTP; protein-tyrosine phosphatase

ROS; reactive oxygen species

RPTP; receptor-like PTP
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Legends to the figures

Fig. 1. **Dose response and kinetics of H$_2$O$_2$-induced disulfide bond formation and dimer stabilization coincide.** After stimulation (for 5 min with 0 - 1 mM H$_2$O$_2$ in $A$ and for 0 - 10 min with 1 mM H$_2$O$_2$ in $B$), cells transfected with both Myc-RPTP$\alpha$ and HA-RPTP$\alpha$ were lysed in the presence of iodoacetamide. Part of the lysate was loaded on non-reducing SDS-PAGE gels (without $\beta$-mercaptoethanol) to investigate intermolecular disulfide bond formation (upper panels) and part was loaded under reducing conditions (with $\beta$-mercaptoethanol) to check expression levels (lower panels). HA-RPTP$\alpha$ was immunoprecipitated from the rest of the lysates and blots were labeled with anti-Myc-antibody to check for co-immunoprecipitation of Myc-RPTP$\alpha$ (middle panels). Immunoblots probed with anti-HA and anti-Myc antibodies, respectively, developed by ECL are depicted.

Fig. 2. **Only catalytic cysteines of RPTP$\alpha$ involved in intermolecular disulfide bond formation.** $A$, cells transfected with both Myc-RPTP$\alpha$ and HA-RPTP$\alpha$ Cys$\rightarrow$Ser mutants were stimulated with 1 mM H$_2$O$_2$ for 5 min as indicated. After lysis, samples from the lysates were taken to check expression levels (lower panel). HA-RPTP$\alpha$ was precipitated from the rest of the lysates and immunoblots were labeled with anti-Myc-antibody to check for co-immunoprecipitation of Myc-RPTP$\alpha$ (upper panel). $B$, cells transfected with an HA-RPTP$\alpha$ Cys$\rightarrow$Ser mutant were stimulated with 0.2 mM H$_2$O$_2$ for 5 min as indicated and lysed in the presence of iodoacetamide. Lysates were loaded on non-reducing SDS-PAGE gels (without $\beta$-mercaptoethanol) to investigate intermolecular disulfide bond formation. Immunoblots developed by ECL are depicted.
Fig. 3. **Cys 723 is required for intermolecular disulfide bond formation leading to covalently linked RPTPα-dimers.** Cells were transfected with both Myc-RPTPα and HA-RPTPα (WT), HA-RPTPα-C433S (C433S), HA-RPTPα-C723S (C723S) or HA-RPTPα-C433S/C723S (C433S/C723S), respectively. After stimulation for 5 min with 0.1 or 1 mM H$_2$O$_2$ as indicated, cells were lysed in the presence of iodoacetamide. Part of the lysate was loaded on non-reducing SDS-PAGE gels (without β-mercaptoethanol) to investigate intermolecular disulfide bond formation (upper panel) and part was loaded under reducing conditions (with β-mercaptoethanol) to check expression levels (lower panel). HA-RPTPα was immunoprecipitated from the rest of the lysates and blots were labeled with anti-Myc-antibody to check for co-immunoprecipitation of Myc-RPTPα (middle panel). Immunoblots probed with anti-HA and anti-Myc antibodies, respectively, developed by ECL are depicted.

Fig. 4. **Catalytic cysteine-dependent intermolecular disulfide bond formation between RPTPα-D2s, but not RPTPα-D1s.** Cells transfected with either Myc-RPTPα-D2 (D2), Myc-RPTPα-D2-C723S (D2-C723S), Myc-RPTPα-D2-C734S (D2-C734S) or Myc-RPTPα-D1 (D1) were stimulated with 1 mM H$_2$O$_2$ for 5 min as indicated. Cells were lysed in the presence of iodoacetamide and loaded under non-reducing conditions (without β-mercaptoethanol, top panel) or under reducing conditions (with β-mercaptoethanol, bottom panel). Gels were blotted, immunoblots were probed with anti-Myc antibody and developed by ECL.
Fig. 5. \( \text{H}_2\text{O}_2 \)-induced rotational coupling and intermolecular disulfide bond formation in RPTP\(\alpha\)-LAR chimera. \) Cells transfected with either HA-RPTP\(\alpha\) (\(\alpha\)-WT) or a chimera of the extracellular domain of HA-RPTP\(\alpha\) with the transmembrane and intracellular domain of LAR (ED\(\alpha\)-LAR), were stimulated for 5 min with 1 mM \( \text{H}_2\text{O}_2 \) as indicated. Subsequently, in A, cells were lysed and the accessible (upper panel) and non-accessible fraction (lower panel) of \( \alpha\)-WT and ED\(\alpha\)-LAR were isolated as described in materials and methods. In B, cells were lysed in the presence of iodoacetamide and loaded on non-reducing SDS-PAGE gels (without \( \beta\)-mercaptoethanol) to investigate intermolecular disulfide bond formation. Immunoblots probed with anti-HA antibody and developed by ECL are depicted.

Fig. 6. Recovery of intermolecular disulfide bond formation and dimer stabilization. \) After stimulation with 1 mM \( \text{H}_2\text{O}_2 \) for 5 min of cells transfected with both HA-RPTP\(\alpha\) and Myc-RPTP\(\alpha\), the medium was replaced and cells were left to recover for 0 - 2h as indicated. Cells were lysed in the presence of iodoacetamide. Part of the lysate was loaded on non-reducing SDS-PAGE gels (without \( \beta\)-mercaptoethanol) to investigate intermolecular disulfide bond formation (upper panel) and part was loaded under reducing conditions (with \( \beta\)-mercaptoethanol) to check expression levels (lower panel). HA-RPTP\(\alpha\) was immunoprecipitated from the rest of the lysates and blots were labeled with anti-Myc-antibody to check for co-immunoprecipitation of Myc-RPTP\(\alpha\) (middle panel). Immunoblots probed with anti-HA and anti-Myc antibodies, respectively, developed by ECL are depicted.

Fig. 7. Differential intermolecular disulfide bond formation and dimer stabilization after \( \text{H}_2\text{O}_2 \), pervanadate and DTT treatment. \) Cells transfected with
both HA-RPTPα and Myc-RPTPα were treated with either 1 mM H₂O₂, 1 mM pervanadate or sequentially with 1 mM H₂O₂ and 1 mM pervanadate or vice versa as indicated. Cells were lysed in the presence of iodoacetamide and 1 mM DTT as indicated. Part of the lysate was loaded on non-reducing SDS-PAGE gels (without β-mercaptoethanol) to investigate intermolecular disulfide bond formation (upper panel) and part was loaded under reducing conditions (with β-mercaptoethanol) to check expression levels (lower panel). HA-RPTPα was immunoprecipitated from the rest of the lysates and blots were labeled with anti-Myc-antibody to check for co-immunoprecipitation of Myc-RPTPα (middle panel). Immunoblots probed with anti-HA and anti-Myc antibodies, respectively, developed by ECL are depicted.

Fig. 8. **Pervanadate triply oxidized RPTPα and did not induce intermolecular disulfide bond formation.** After stimulation with either 1 mM H₂O₂ or 1 mM pervanadate as indicated, cells were lysed in the presence of iodoacetamide. Part of the lysate was loaded on non-reducing SDS-PAGE gels (without β-mercaptoethanol) to investigate intermolecular disulfide bond formation (upper panel). HA-RPTPα was immunoprecipitated from the rest of the lysates and blots were labeled with anti-oxPTP antibody (middle panel) and anti-RPTPα antibody (lower panel), respectively. Immunoblots developed by ECL are depicted.
van der Wijk et al., figure 1
van der Wijk et al., figure 2

A

|          | Cys mutant | WT | 242 | 248 | 339 | 341 | 350 | 375 | 421 | 433 | 473 | 618 | 634 | 659 | 723 | 775 |
|----------|------------|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|          |            | -  | +   | +   | +   | -   | +   | +   | +   | +   | +   | +   | -   | +   | -   | +   |
|          |            | +  | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |

IP: anti-HA-Ab
Blot: anti-Myc-Ab

WCL
Blot: anti-Myc-Ab

B

|          | Cys mutant | WT | 242 | 248 | 339 | 341 | 350 | 375 | 421 | 433 | 473 | 618 | 634 | 659 | 723 | 775 |
|----------|------------|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|          |            | -  | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
|          |            | +  | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |

WCL: non-reducing
Blot: anti-HA-Ab
van der Wijk et al., figure 3

| Construct     | H$_2$O$_2$ (mM) | Blot: anti-HA-Ab | Blot: anti-Myc-Ab |
|---------------|-----------------|------------------|------------------|
| WT            | - 0.1 1         | WCL, non-reducing| IP: anti-HA-Ab   |
| C433S         | - 0.1 1         | Blot: anti-HA-Ab | Blot: anti-Myc-Ab|
| C723S         | - 0.1 1         | WCL              | WCL              |
| C433S/C723S   | - 0.1 1         |                  |                  |
van der Wijk et al., figure 4
van der Wijk et al., figure 5

**A**

| Accessible |  |  |
|------------|---|---|
| Non-accessible |  |  |

| H₂O₂ | - | + | - | + |
| construct | α-WT | EDα-LAR | α-WT | EDα-LAR |

**B**

| H₂O₂ | - | + | - | + | - | + |
| construct | α-WT | EDα-LAR | α-WT | EDα-LAR |

WCL blot: anti-HA-Ab
- Non-reducing
- Reducing
van der Wijk et al., figure 6

| WCL: non-reducing Blot: anti-HA-Ab | 
|---|---|
| dimer | monomer |

| IP: anti-HA-Ab Blot: anti-Myc-Ab |
|---|---|

| WCL Blot: anti-Myc-Ab |
|---|---|

| H$_2$O$_2$ (mM) | - | + | + | + | + | + |
|---|---|---|---|---|---|---|

| Recovery (min) | 15 | 30 | 60 | 90 | 120 |
|---|---|---|---|---|---|

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van der Wijk et al., figure 7

| WCL: non-reducing | dimer | monomer |
|-------------------|-------|---------|
| Blot: anti-HA-Ab  |       |         |

| IP: anti-HA-Ab     |       |         |
| Blot: anti-Myc-Ab  |       |         |

| WCL               |       |         |
| Blot: anti-Myc-Ab |       |         |

| pre-treatment     |       | H2O2 Va |       |       |
| treatment         |       | H2O2 Va | H2O2 | H2O2 Va |
| DTT in lysis      |       | + + + + |       |       |
van der Wijk et al., figure 8

| WCL: non-reducing Blot: anti-HA-Ab | dimer |
|-----------------------------------|-------|
|                                   | monomer |

| IP: anti-HA-Ab                  | Blot: anti-oxPTP-Ab     |
|---------------------------------|-------------------------|

| IP: anti-HA-Ab                  | Blot: anti-RPTPα-Ab     |
|---------------------------------|-------------------------|

| treatment | - | H₂O₂ | Va    |
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