THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 43, Issue of October 22, pp. 44355–44361, 2004

Printed in U.S.A.

H_2O_2-induced Intermolecular Disulfide Bond Formation between Receptor Protein-tyrosine Phosphatases*

Received for publication, July 6, 2004
Published, JBC Papers in Press, August 4, 2004, DOI 10.1074/jbc.M407483200

Thea van der Wijk, John Overvoorde, and Jeroen den Hertog‡

From the Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

Receptor protein-tyrosine phosphatase α (RPTPα) 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 H_2O_2 on non-reducing SDS-polyacrylamide 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 protein-tyrosine phosphatase domains, we located the intermolecular disulfide bond to the catalytic Cys-723 in D2. Disulfide bond formation and dimer stabilization showed similar levels of concentration and time dependence. However, treatment of lysates with dithiothreitol 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 the leukocyte common antigen-related protein (LAR). These results suggest that H_2O_2 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.

Protein-tyrosine phosphatases (PTPs)[1] form a family of enzymes that catalyze 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 (for review, see 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 pK_a of the catalytic cysteine, PTPs are very susceptible to oxidation (for review, see Ref. 4). Reactive oxygen species induce oxidation of catalytic cysteines, thereby inactivating these PTPs (5–8). Extracellular stimuli like growth factors and UV irradiation result in an increase in intracellular reactive oxygen species 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_2O_2 treatment induces a rapid, reversible, and catalytic site Cys-723-dependent *in vivo* change in protein conformation, rendering a more stable, catalytically inactive RPTPα dimer (22).

Low molecular weight PTP (LMW-PTP), CDC25, and phosphatase and tensin homolog (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_2O_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 are not restricted to RPTPα but are a general theme for RPTPs with two intracellular PTP domains.

* This work was supported in part by the Dutch Cancer Society/Koningin Wilhelmina Fonds. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 31-302121800; Fax: 31-302516464; E-mail: hertog@niob.knaw.nl.

[1] The abbreviations used are: PTP, protein-tyrosine phosphatase; DTT, dithiothreitol; ED, extracellular domain; FRET, fluorescence resonance energy transfer technique; HA, hemagglutinin; LAR, leukocyte common antigen-related; RPTP, receptor-like PTP.

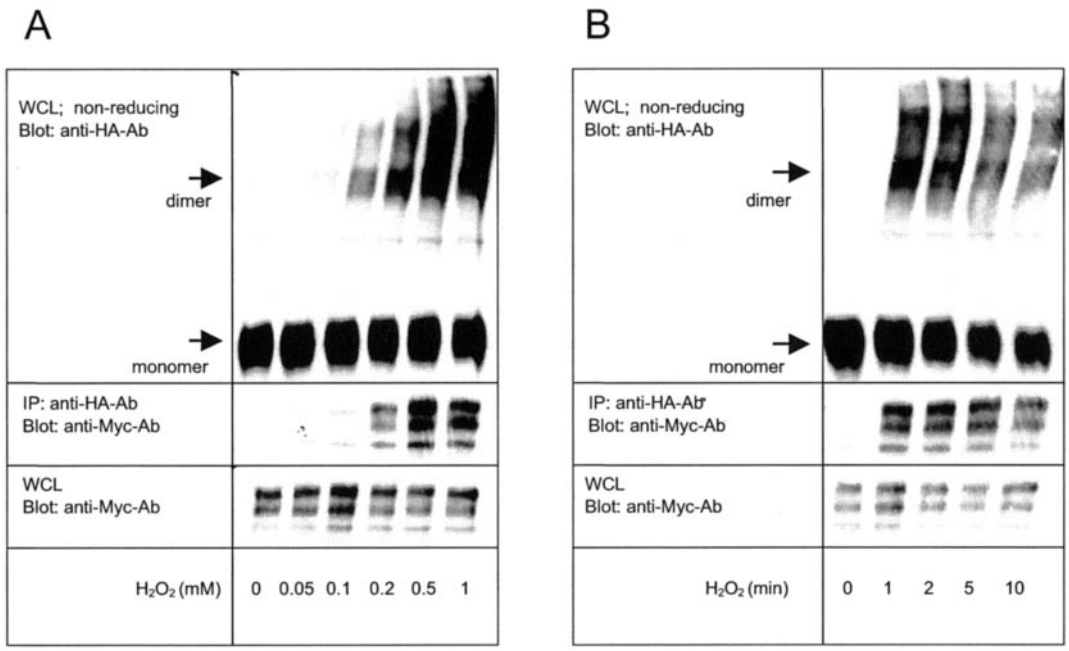

FIG. 1. **Dose response and kinetics of H_2O_2-induced disulfide bond formation and dimer stabilization coincide.** After stimulation (for 5 min with 0–1 mM H_2O_2 in *A* and for 0–10 min with 1 mM H_2O_2 in *B*), cells transfected with both Myc-RPTPα and HA-RPTPα were lysed in the presence of iodoacetamide. Part of the lysate was loaded on non-reducing SDS-polyacrylamide gels (without β-mercaptoethanol) to investigate intermolecular disulfide bond formation (*upper panels*), and part was loaded under reducing conditions (with β-mercaptoethanol) to check expression levels (*lower panels*). 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 panels*). Immunoblots probed with anti-HA and anti-Myc antibodies, respectively, developed by ECL are depicted. *WCL*, whole cell lysate; *Ab*, antibody; *IP*, immunoprecipitation.

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 previously 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 RPTPα-D1 and RPTPα-D2 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 Dulbecco's modified Eagle's medium/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 H_2O_2 (Sigma) 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 $MgCl_2$, 10% glycerol, 1% Triton X-100, aprotinin, and leupeptin) in the presence of 20 mM iodoacetamide when indicated and centrifuged at 14,000 × *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 2× 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-polyacrylamide 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-polyacrylamide gels. The material on the gel was transferred to polyvinylidene difluoride membrane by semidry 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 h with anti-HA, anti-Myc, or anti-RPTPα antibody 5478 (26) in TBST + 5% milk. The blots were washed three times with TBST and incubated with horseradish peroxidase-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 has been described previously in detail (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 Disulfide Bonds between RPTPα Monomers in Response to H_2O_2—Previously, we showed that H_2O_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_2O_2 for 5 min (Fig. 1*A*). The cells were lysed, and samples were run on SDS-polyacrylamide gels under reducing and non-reducing conditions. HA-RPTPα was immunoprecipitated from

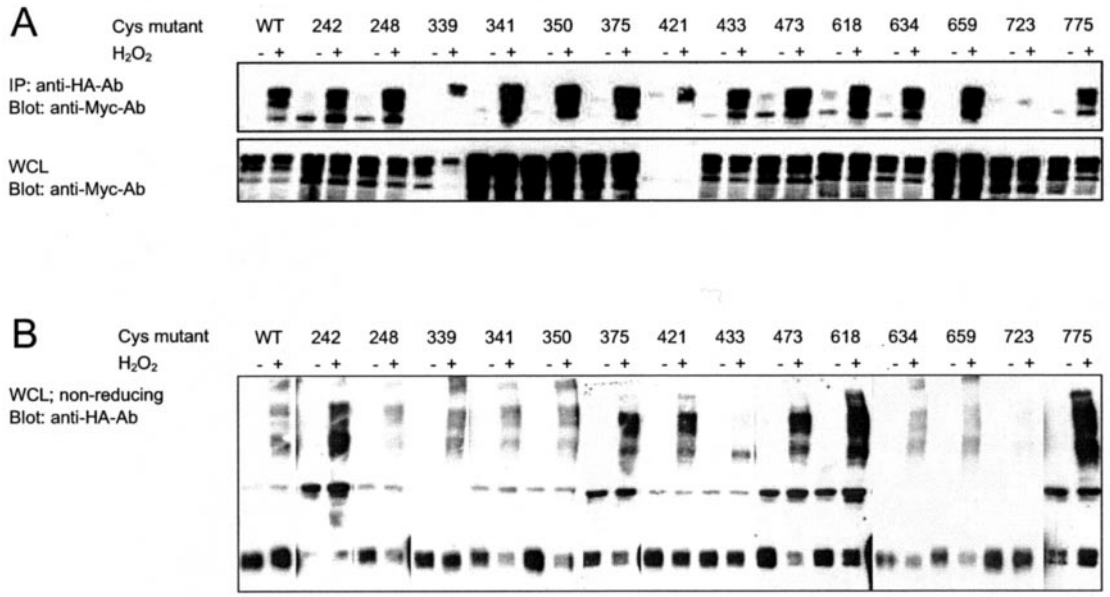

FIG. 2. **Only catalytic cysteines of RPTPα are involved in intermolecular disulfide bond formation.** *A*, cells transfected with both Myc-RPTPα and HA-RPTPα Cys → Ser mutants were stimulated with 1 mM H_2O_2 for 5 min as indicated. After lysis, samples from the lysates were taken to check expression levels (*bottom*). HA-RPTPα was precipitated from the rest of the lysates, and immunoblots were labeled with anti-Myc antibody to check for co-immunoprecipitation of Myc-RPTPα (*top*). *WT*, wild type; *IP*, immunoprecipitation; *Ab*, antibody; *WCL*, whole cell lysate. *B*, cells transfected with an HA-RPTPα Cys → Ser mutant were stimulated with 0.2 mM H_2O_2 for 5 min as indicated and lysed in the presence of iodoacetamide. Lysates were loaded on non-reducing SDS-polyacrylamide gels (without β-mercaptoethanol) to investigate intermolecular disulfide bond formation. Immunoblots developed by ECL are depicted.

the remaining lysates, and co-immunoprecipitation of Myc-tagged RPTPα was detected. H_2O_2 treatment induced RPTPα in a dose-dependent manner to migrate at a position that corresponds to the double molecular weight of an RPTPα monomer on SDS-polyacrylamide gels under non-reducing conditions (Fig. 1*A*, *upper panel*). The RPTPα bands that appeared on SDS-polyacrylamide gels under non-reducing conditions after H_2O_2 stimulation were absent under reducing conditions (data not shown, *cf.* Fig. 5*B*). As shown in the *middle panel* of Fig. 1*A*, the increasing amount of RPTPα dimers under non-reducing SDS-PAGE conditions coincided with the co-immunoprecipitation of Myc-tagged RPTPα with HA-RPTPα.

Fig. 1*B* shows that dimer formation detected by SDS-PAGE under non-reducing conditions and dimer formation detected by co-immunoprecipitation of differentially tagged RPTPα constructs coincided in time. Dimers were already prominently and maximally formed after 1 min of H_2O_2 treatment and declined slightly after ~5 min. These results show that intermolecular disulfide bonds are formed between RPTPα monomers with similar kinetics as stable co-immunoprecipitating RPTPα dimers.

Intermolecular Disulfide Bonds between Catalytic Cysteines of RPTPα—To map the cysteines involved in H_2O_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_2O_2 or left untreated. Cells were lysed, and samples were taken to run on SDS-polyacrylamide 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 Fig. 2*A*, Myc-RPTPα co-precipitated with all but one cysteine to serine mutant; only Cys-723 was found to be essential for the H_2O_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 and C421S), they still showed appreciable co-immunoprecipitation with Myc-RPTPα. Note that the RPTPα-C734S is lacking because expression was undetectable. Fig. 2*B* demonstrates that only the C433S and C723S mutant showed a decreased amount of intermolecular disulfide bond formation upon peroxide treatment, whereas 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 HA-tagged wild-type RPTPα, HA-tagged RPTPα-C433S, HA-tagged RPTPα-C723S, or the double mutant HA-tagged RPTPα-C433S/C723S. The C433S mutant behaved like wild type with respect to H_2O_2-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-polyacrylamide gels. However, a band that migrated slower than the expected dimeric RPTPα band was still present in the C723S mutant after H_2O_2 treatment (Fig. 3). Interestingly, this band was strongly reduced in cells transfected with HA-RPTPα-C433S or HA-RPTPα-C433S/C723S in response to H_2O_2, suggesting that the catalytic cysteine of the RPTPα-D1 is involved in the formation of this slower migrating complex.

Using constructs encoding the individual catalytic domains D1 and D2, respectively, we found that only D2 forms intermolecular disulfide-bonded dimers that migrate in SDS-polyacrylamide gels under non-reducing conditions with an apparent molecular weight of D2-D2 homodimers, which was completely dependent on Cys-723 (Fig. 4). D1 did not form intermolecular disulfide bonds upon treatment of cells with 1 mM H_2O_2 (Fig. 4). Because full-length RPTPα-C734S was not expressed in cells, we assessed involvement of Cys-734 in intermolecular disulfide bond formation in the D2 domain by itself. Importantly, D2-C734S acted in a similar fashion as

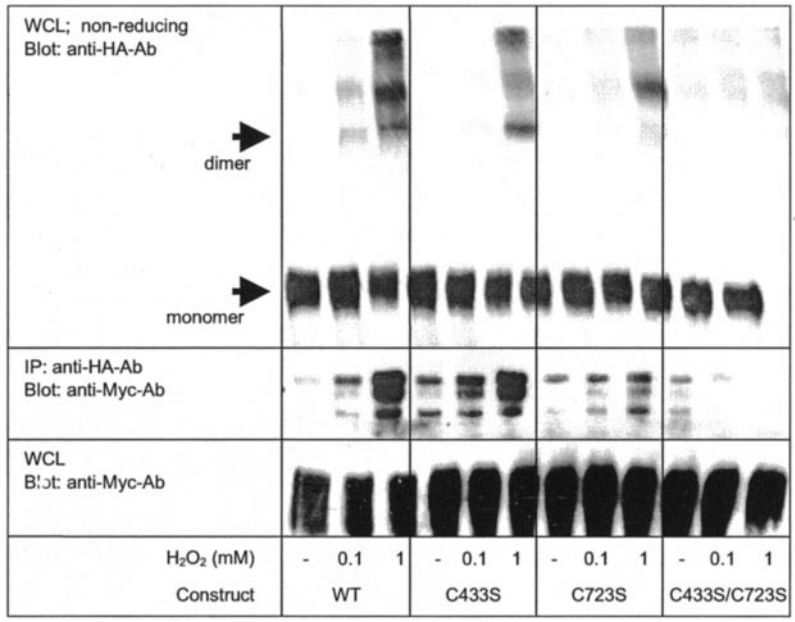

FIG. 3. **Cys-723 is required for intermolecular disulfide bond formation leading to covalently linked RPTPα dimers.** Cells were transfected with both Myc-RPTPα and HA-RPTPα (wild type (*WT*)), HA-RPTPα-C433S (*C433S*), HA-RPTPα-C723S (*C723S*), or HA-RPTPα-C433S/C723S (*C433S/C723S*), respectively. After stimulation for 5 min with 0.1 or 1 mM H_2O_2 as indicated, cells were lysed in the presence of iodoacetamide. Part of the lysate was loaded on non-reducing SDS-polyacrylamide gels (without β-mercaptoethanol) to investigate intermolecular disulfide bond formation (*top*), and part was loaded under reducing conditions (with β-mercaptoethanol) to check expression levels (*bottom*). 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*). Immunoblots probed with anti-HA and anti-Myc antibodies, respectively, developed by ECL are depicted. *WCL*, whole cell lysate; *Ab*, antibody; *IP*, immunoprecipitation.

wild-type D2 indicating that Cys-734 is not involved in intermolecular disulfide bond formation. These results suggest that upon H_2O_2 treatment, the catalytic Cys-723 forms a disulfide bond, cross-linking RPTPα-D2s.

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 H_2O_2 treatment as detected by the accessibility of the HA tag on living cells transfected with HA-EDα-LAR (Fig. 5*A*). As shown in Fig. 5*B*, H_2O_2 induced HA-EDα-LAR to migrate at a level that corresponds to the double molecular weight of an HA-EDα-LAR dimer on SDS-polyacrylamide 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—Because Cys-723 is required both for intermolecular disulfide bond formation and for stable dimer formation as detected by co-immunoprecipitation of differentially tagged RPTPα monomers and because both emerge with the same kinetics in response to H_2O_2 treatment (Fig. 1*B*), 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 H_2O_2-induced RPTPα dimer formation indicated a difference in persistence of the intermolecular disulfide bonds and RPTPα dimer stabilization. Cells transfected with wild-type RPTPα were treated for 5 min with 1 mM H_2O_2. The medium was replaced with medium without H_2O_2, and the cells were left to recover for 0–2 h. Fig. 6 shows that intermolecular disulfide bonds were less persistent than co-immunoprecipitating RPTPα dimers; although high molecular weight bands on SDS-polyacrylamide 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 H_2O_2 and lysed the cells in the presence or absence of 1 mM dithiothreitol (DTT) (Fig. 7). We found that reduction with DTT abolished the intermolecular disulfide bonds, whereas 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 Fig. 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-ox-PTP (12). As shown in Fig. 8, 1 mM pervanadate indeed led to the formation of triply oxidized RPTPα, whereas no triply oxidized RPTPα is formed after 5 min of treatment with 1 mM H_2O_2. Interestingly, when cells are first treated with H_2O_2 for 5 min and subsequently with 1 mM pervanadate for 5 min, stable dimers and intermolecular disulfide bonds are formed comparable with treatment with H_2O_2 alone, indicating that disulfide bond formation protects against irreversible oxidation. Conversely, when cells are first stimulated with pervanadate and then with H_2O_2, 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 sulfenic 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 sulfenyl-amide after oxidation by H_2O_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 sulfenyl-amide results in a rapid elimination of oxygen from sulfenic acid and suppresses further irreversible oxidation. In this paper we show that RPTPα forms intermolecular disulfide bonds between the catalytic cysteines of the membrane-distal PTP domain upon treatment of cells with H_2O_2.

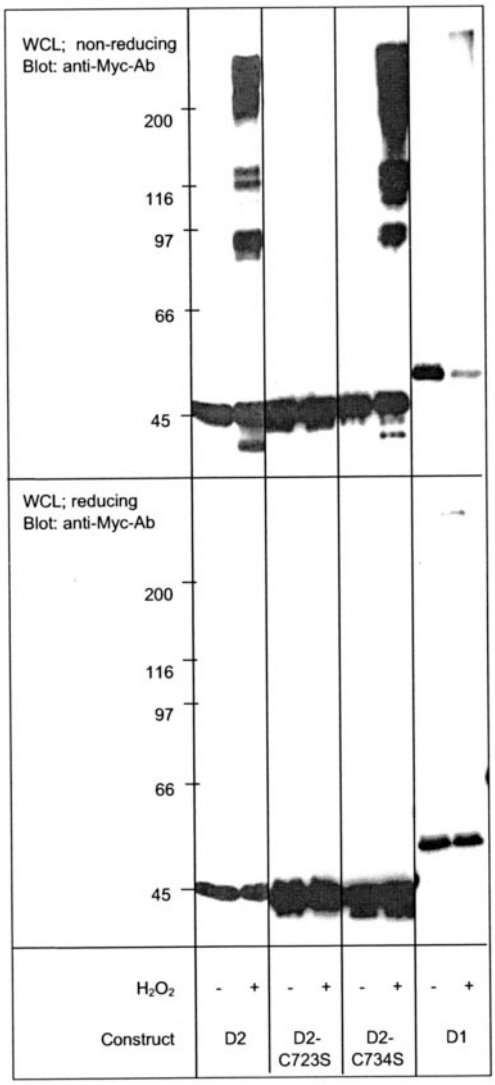

FIG. 4. **Catalytic cysteine-dependent intermolecular disulfide bond formation between RPTPα-D2s but not RPTPα-D1s.** Cells transfected with Myc-RPTPα-D2 (*D2*), Myc-RPTPα-D2-C723S (*D2-C723S*), Myc-RPTPα-D2-C734S (*D2-C734S*), or Myc-RPTPα-D1 (*D1*) were stimulated with 1 mM H_2O_2 for 5 min as indicated. Cells were lysed in the presence of iodoacetamide and loaded under non-reducing conditions (without β-mercaptoethanol, *top*) or under reducing conditions (with β-mercaptoethanol, *bottom*). Gels were blotted, and immunoblots were probed with anti-Myc antibody and developed by ECL. *WCL*, whole cell lysate; *Ab*, antibody.

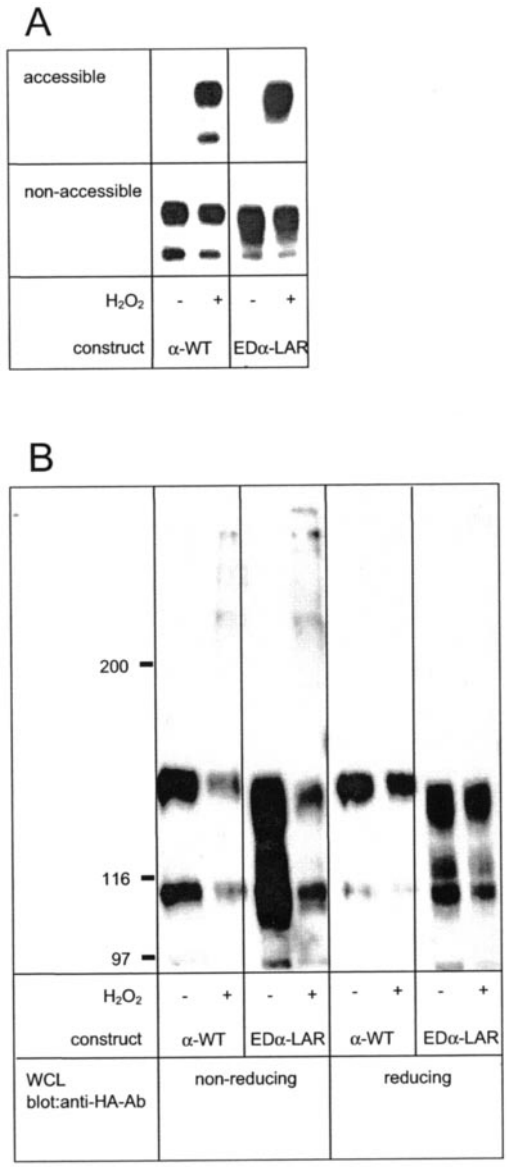

FIG. 5. **H_2O_2-induced rotational coupling and intermolecular disulfide bond formation in RPTPα-LAR chimera.** Cells transfected with either HA-RPTPα (*α-WT*) or a chimera of the extracellular domain of HA-RPTPα with the transmembrane and intracellular domain of LAR (*EDα-LAR*) were stimulated for 5 min with 1 mM H_2O_2 as indicated. Subsequently, in *A*, cells were lysed, and the accessible fraction (*top*) and non-accessible fraction (*bottom*) of α-WT and EDα-LAR were isolated as described under "Materials and Methods." In *B*, cells were lysed in the presence of iodoacetamide and loaded on non-reducing SDS-polyacrylamide gels (without β-mercaptoethanol) to investigate intermolecular disulfide bond formation. Immunoblots probed with anti-HA antibody and developed by ECL are depicted. *WCL*, whole cell lysate; *Ab*, antibody.

Recently, by using a conformation-sensitive antibody method, we found that H_2O_2 treatment leads to a change in rotational coupling of the RPTPα dimers that is dependent on the catalytic Cys-723 of the membrane-distal PTP domain (27). The conformation of untreated RPTPα dimers is similar to the conformation of the active constitutive dimer mutant RPTPα-F135C, whereas the H_2O_2-treated RPTPα dimers adopt the conformation as found with an inactive constitutive dimer mutant, RPTPα-P137C. The H_2O_2-induced intermolecular disulfide bond formation, like stabilized dimer formation and rotational coupling of RPTPα dimers, depends on the catalytic cysteine of the membrane-distal PTP domain. Furthermore, intermolecular disulfide bonds and stable dimers are formed with similar kinetics upon treatment of cells with H_2O_2. These results suggest that intermolecular disulfide bond formation between the Cys-723s of the RPTPα 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_2O_2-induced conformational changes of RPTPα-D2 were studied using the fluorescence resonance energy transfer technique (FRET; Ref. 22). A decrease in FRET was found upon H_2O_2 treatment of lysates containing the cyan fluorescent protein (CFP)-RPTPα-D2-yellow fluorescent protein (YFP) construct. Interestingly, treatment of cell lysates with H_2O_2 did not lead to the formation of intermolecular disulfide bonds between RPTPα 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

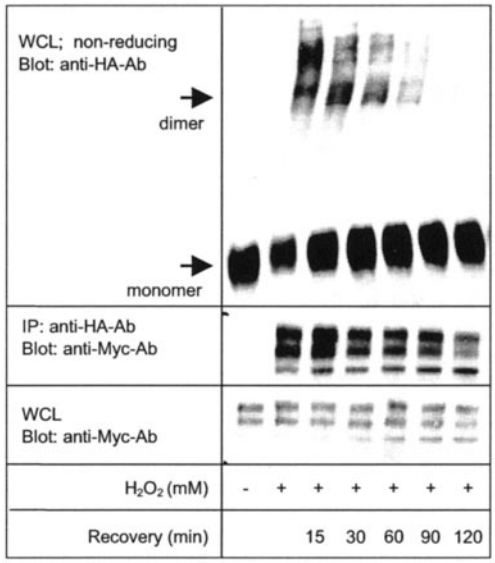

FIG. 6. **Recovery of intermolecular disulfide bond formation and dimer stabilization.** After stimulation of cells transfected with both HA-RPTPα and Myc-RPTPα with 1 mM H_2O_2 for 5 min, the medium was replaced, and cells were left to recover for 0–2 h as indicated. Cells were lysed in the presence of iodoacetamide. Part of the lysate was loaded on non-reducing SDS-polyacrylamide gels (without β-mercaptoethanol) to investigate intermolecular disulfide bond formation (*top*), and part was loaded under reducing conditions (with β-mercaptoethanol) to check expression levels (*bottom*). 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*). Immunoblots probed with anti-HA and anti-Myc antibodies, respectively, developed by ECL are depicted. *WCL*, whole cell lysate; *Ab*, antibody; *IP*, immunoprecipitation.

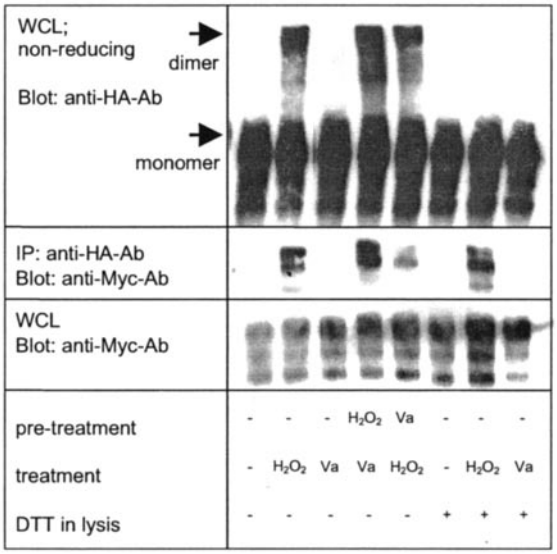

FIG. 7. **Differential intermolecular disulfide bond formation and dimer stabilization after H_2O_2, pervanadate, and DTT treatment.** Cells transfected with both HA-RPTPα and Myc-RPTPα were treated with either 1 mM H_2O_2 or 1 mM pervanadate, sequentially with 1 mM H_2O_2 and 1 mM pervanadate, or *vice 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-polyacrylamide gels (without β-mercaptoethanol) to investigate intermolecular disulfide bond formation (*top*), and part was loaded under reducing conditions (with β-mercaptoethanol) to check expression levels (*bottom*). 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*). Immunoblots probed with anti-HA and anti-Myc antibodies, respectively, developed by ECL are depicted. *WCL*, whole cell lysate; *Ab*, antibody; *IP*, immunoprecipitation; *Va*, pervanadate.

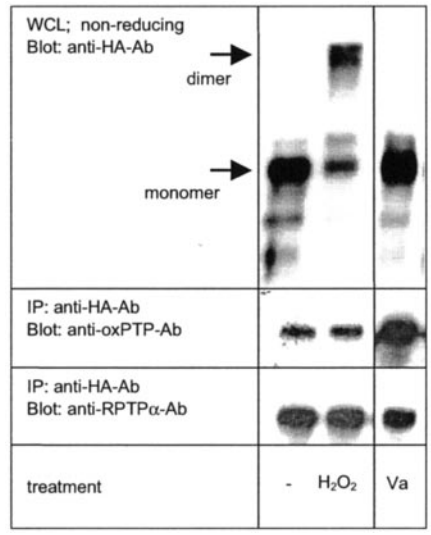

FIG. 8. **Pervanadate triply oxidized RPTPα and did not induce intermolecular disulfide bond formation.** After stimulation with either 1 mM H_2O_2 or 1 mM pervanadate as indicated, cells were lysed in the presence of iodoacetamide. Part of the lysate was loaded on non-reducing SDS-polyacrylamide gels (without β-mercaptoethanol) to investigate intermolecular disulfide bond formation (*top*). HA-RPTPα was immunoprecipitated from the rest of the lysates, and blots were labeled with anti-oxPTP antibody (*middle*) and anti-RPTPα antibody (*bottom*), respectively. Immunoblots developed by ECL are depicted. *WCL*, whole cell lysate; *Ab*, antibody; *IP*, immunoprecipitation; *Va*, pervanadate.

results rule out the possibility that the H_2O_2-induced conformational change in RPTPα-D2 is caused by the formation of disulfide bonds between RPTP-D2s. It is likely that the reversible conformational change in RPTPα-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 Cys-723 from the dyad-related RPTPα 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_2O_2 immediately reduced the intermolecular disulfide bonds between RPTPα monomers, whereas differentially tagged monomers still co-immunoprecipitated. In line with this, recovery from the H_2O_2-induced conformational changes of RPTPα-D2 in cells as detected by FRET was relatively slow (22), indicating that refolding of RPTPα-D2 is independent of disulfide bond reduction but probably a result of another intrinsic property of the protein.

Taken together, these findings led us to the model that H_2O_2-induced oxidation of the RPTPα-Cys-723 to cyclic sulfenyl-amide leads to a conformational change of RPTPα-D2s and thereby a change in rotational coupling resulting in dimer stabilization. This cyclic sulfenyl-amide reacts rapidly with the thiolate anion Cys-723 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.

Acknowledgment—We thank Arne Östman (Stockholm, Sweden) for the oxPTP antibody.

REFERENCES

1. Andersen, J. N., Mortensen, O. H., Peters, G. H., Drake, P. G., Iversen, L. F., Olsen, O. H., Jansen, P. G., Andersen, H. S., Tonks, N. K., and Moller, N. P. (2001) *Mol. Cell. Biol.* **21,** 7117–7136
2. Alonso, A., Sasin, J., Bottini, N., Friedberg, I., Friedberg, I., Osterman, A., Godzik, A., Hunter, T., Dixon, J., and Mustelin, T. (2004) *Cell* **117,** 699–711
3. Barford, D., Jia, Z., and Tonks, N. K. (1995) *Nat. Struct. Biol.* **2,** 1043–1053
4. den Hertog, J., Groen, A., and Van der Wijk, T. (2004) *Arch. Biochem. Biophys.*, in press
5. Lee, S. R., Kwon, K. S., Kim, S. R., and Rhee, S. G. (1998) *J. Biol. Chem.* **273,** 15366–15372
6. Denu, J. M., and Tanner, K. G. (1998) *Biochemistry* **37,** 5633–5642
7. Caselli, A., Marzocchini, R., Camici, G., Manao, G., Moneti, G., Pieraccini, G., and Ramponi, G. J. (1998) *Biol. Chem.* **273,** 32554–32560
8. Lee, S. R., Yang, K. S., Kwon, J., Lee, C., Jeong, W., and Rhee, S. G. (2002) *J. Biol. Chem.* **277,** 20336–20342
9. Gross, S., Knebel, A., Tenev, T., Neininger, A., Gaestel, M., Herrlich, P., and Bohmer, F. D. (1999) *J. Biol. Chem.* **274,** 26378–26386
10. Mahadev, K., Zibering, A., Zhu, L., and Goldstein, B. J. (2001) *J. Biol. Chem.* **276,** 21938–21942
11. Meng, T. C., Fukada, T., and Tonks, N. K. (2002) *Mol. Cell* **9,** 387–399
12. Persson, C., Sjöblom, T., Groen, A., Kappert, K., Engström, U., Hellman, U., Heldin, C. H., den Hertog, J., and Östman, A. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 1886–1891
13. Jiang, G., den Hertog, J., and Hunter, T. (2000) *Mol. Cell. Biol.* **20,** 5917–5929
14. Tertoolen, L. G., Blanchetot, C., Jiang, G., Overvoorde, J., Gadella, T. W., Jr., Hunter, T., and den Hertog, J. (2001) *BMC Cell Biol.* http://www.biomedcentral.com/1471-2121/2/8
15. Jiang, G., den Hertog, J., Su, J., Noel, J., Sap, J., and Hunter, T. (1999) *Nature* **401,** 606–610
16. Bilwes, A. M., den Hertog, J., Hunter, T., and Noel, J. P. (1996) *Nature* **382,** 555–559
17. Desai, D. M., Sap, J., Schlessinger, J., and Weiss, A. (1993) *Cell* **73,** 541–554
18. Xu, Z., and Weiss, A. (2002) *Nat. Immunol.* **3,** 764–771
19. Majeti, R., Bilwes, A. M., Noel, J. P., Hunter, T., and Weiss, A. (1998) *Science* **279,** 88–91
20. Blanchetot, C., and den Hertog, J. (2000) *J. Biol. Chem.* **275,** 12446–12452
21. Blanchetot, C., Tertoolen, L. G., Overvoorde, J., and den Hertog, J. (2002) *J. Biol. Chem.* **277,** 47263–47269
22. Blanchetot, C., Tertoolen, L. G., and den Hertog, J. (2002) *EMBO J.* **21,** 493–503
23. Savitsky, P. A., and Finkel, T. (2002) *J. Biol. Chem.* **277,** 20535–20540
24. den Hertog, J., and Hunter, T. (1996) *EMBO J.* **15,** 3016–3027
25. Buist, A., Blanchetot, C., and den Hertog, J. (2000) *Biochem. Biophys. Res. Commun.* **267,** 96–102
26. den Hertog, J., Tracy, S., and Hunter, T. (1994) *EMBO J.* **13,** 3020–3032
27. Van der Wijk, T., Blanchetot, C., Overvoorde, J., and den Hertog, J. (2003) *J. Biol. Chem.* **278,** 13968–13974
28. Huyer, G., Lui, S., Kelly, J., Moffat, J., Payette, P., Kennedy, B., Tsaprailis, G., Gresser, M. J., and Ramachandran, C. (1997) *J. Biol. Chem.* **272,** 843–851
29. Salmeen, A., Andersen, J. N., Myers, M. P., Meng, T. C., Hinks, J. A., Tonks, N. K., and Barford, D. (2003) *Nature* **423,** 769–773
30. van Montfort, R. L., Congreve, M., Tisi, D., Carr, R., and Jhoti, H. (2003) *Nature* **423,** 773–777

H_2O_2-induced Intermolecular Disulfide Bond Formation between Receptor Protein-tyrosine Phosphatases

Thea van der Wijk, John Overvoorde and Jeroen den Hertog

J. Biol. Chem. 2004, 279:44355-44361.
doi: 10.1074/jbc.M407483200 originally published online August 4, 2004
