Redox regulation of dimerization of the receptor protein-tyrosine phosphatases RPTPα, LAR, RPTPμ and CD45

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Whether dimerization is a general regulatory mechanism of receptor protein-tyrosine phosphatases (RPTPs) is a subject of debate. Biochemical evidence demonstrates that RPTPα and cluster of differentiation (CD)45 dimerize. Their catalytic activity is regulated by dimerization and structural evidence from RPTPα supports dimerization-induced inhibition of catalytic activity. The crystal structures of CD45 and leukocyte common antigen related (LAR) indicate that dimerization would result in a steric clash. Here, we investigate dimerization of four RPTPs. We demonstrate that LAR and RPTPμ dimerized constitutively, which is likely to be due to their ectodomains. To investigate the role of the cytoplasmic domain in dimerization we generated RPTPα ectodomain (EDα)/RPTP chimeras and found that – similarly to native RPTPα – oxidation stabilized their dimerization. Limited tryptic proteolysis demonstrated that oxidation induced conformational changes in the cytoplasmic domains of these RPTPs, indicating that the cytoplasmic domains are not rigid structures, but rather that there is flexibility. Moreover, oxidation induced changes in the rotational coupling of dimers of full length EDα/RPTP chimeras in living cells, which were largely dependent on the catalytic cysteine in the membrane-distal protein-tyrosine phosphatase domain of RPTPα and LAR. Our results provide new evidence for redox regulation of dimerized RPTPs.

Phosphorylation on tyrosine residues is of major importance in cell signalling and regulates processes like cell migration, cell proliferation and cell differentiation. Therefore, the balance in tyrosine phosphorylation, mediated by protein-tyrosine kinases (PTKs), and dephosphorylation, mediated by protein-tyrosine phosphatases (PTPs), must be tightly controlled [1]. PTKs and PTPs have important roles in diseases like cancer and diabetes.

The human genome encodes 21 classical PTPs with a transmembrane domain [2,3]. Most of these receptor protein-tyrosine phosphatases (RPTPs) have two intracellular PTP domains. The membrane proximal domain (D1) contains most catalytic activity, whereas the membrane distal domain (D2) has a regulatory function [4]. Ligands have been identified that bind to the ectodomain of RPTPs. Ligand binding may regulate RPTP catalytic activity. For instance, Pleiotrophin binds RPTPβ/ζ and regulates its activity [5].

RPTPs are regulated by various mechanisms, including dimerization. Structural evidence indicates that dimerization inhibits RPTPα catalytic activity due to a helix-loop-helix wedge interaction of one molecule with the catalytic site of the other molecule in dimers [6]. We have demonstrated that RPTPα dimerizes constitutively in living cells using fluorescence resonance energy transfer [7] and using cross-linkers [8]. Not only RPTPs, but also fragments of RPTPs homo- and

heterodimerize [9–11]. Dimeric mutants with disulfide bonds in their ectodomain are catalytically active or inactive, depending on the exact location of the disulfide bond, indicating that rotational coupling within the dimers is crucial for RPTPα activity [12,13]. Cluster of differentiation (CD)45 also forms dimers [14,15] and an epidermal growth factor receptor (EGFR)-CD45 chimera is functionally inactivated by EGF-induced dimerization [16], which is dependent on the wedge of CD45 [17,18]. However, the crystal structures of CD45 and leukocyte common antigen related (LAR) are not compatible with dimerization-induced inactivation caused by wedge-catalytic site interactions, due to a steric clash with their D2s [19,20]. Nevertheless, the inactive conformation might form if there were flexibility between D1 and D2.

Regulation of PTPs by oxidation is emerging as an important regulatory mechanism [21]. Reactive oxygen species (ROS) are produced in response to physiological stimuli [22–24] and oxidation of PTP1B enhances signaling [25,26]. The catalytic site cysteines of PTPs are highly susceptible to oxidation due to their low pKa [27]. Oxidation of PTP1B results in cyclic sulfenamide formation, which is reversible, inactivates the PTP, and protects the cysteine from irreversible double or triple oxidation [28,29]. We found that the catalytic cysteine of RPTPα-D2 is more susceptible to oxidation than RPTPα-D1 [30] and, in general, that PTPs are differentially oxidized [31]. Interestingly, these data are consistent with functional data which show that RPTPα-D2 is important for the effects of oxidation and acts as a redox sensor [8,13,32]. Oxidation of RPTPα-D2, like PTP1B, results in the formation of cyclic sulfenamide at the catalytic site, which is stable and reversible by thiols [33].

The model that is emerging for regulation of RPTPα suggests that it dimerizes constitutively (for a recent review see [34]). Depending on the quaternary structure, RPTPα dimers are in the open (active) or closed (inactive) conformation. Oxidation or other stimuli may drive RPTPα dimers into the closed (inactive) conformation. Here, we investigated dimerization and the role of oxidation in dimerization of a panel of four different RPTPs.

We compared four RPTPs from different subtypes, i.e. RPTPα, RPTPμ, CD45 and LAR. We found that the cytoplasmic domains of these RPTPs may contribute to dimerization upon oxidation. Limited tryptic proteolysis indicated an oxidation-induced conformational change in the cytoplasmic domains and oxidation induced a change in rotational coupling of chimeric receptors, suggesting that this panel of dimerized RPTPs is regulated in a similar manner.

Results

To investigate whether dimerization is a common mechanism for RPTPs, we assayed dimerization by co-immunoprecipitation of three different RPTPs, i.e. LAR, RPTPμ and, used as a control, RPTPα. Dimerization of full length CD45, the fourth RPTP that we investigated here, has been established previously [14,15]. Cos-1 cells were co-transfected with Myc-tagged and hemagglutinnin (HA)-tagged RPTP constructs. Cells were left untreated or were incubated with 0.1 mM or 1 mM H_2O_2 for 5 min. Myc-tagged LAR co-immunoprecipitated with HA-tagged LAR in the absence or presence of H_2O_2 (Fig. 1A). Likewise, Myc-tagged RPTPμ co-immunoprecipitated constitutively with HA-tagged RPTPμ (Fig. 1B). RPTPα dimerized constitutively as detected by fluorescence resonance energy transfer and using cross-linkers [7,8]. As described previously [8], Myc-tagged RPTPα co-immunoprecipitated with HA-tagged RPTPα only after treatment with H_2O_2 (Fig. 1C). Apparently, the binding affinity within RPTPα dimers is too low to detect dimerization by co-immunoprecipitation under control conditions and the binding affinity increases upon H_2O_2-treatment. Taken together, we demonstrate here that LAR and RPTPμ co-immunoprecipitated constitutively, whereas RPTPα co-immunoprecipitated only after H_2O_2 treatment.

The extensive ectodomains of LAR and RPTPμ may drive homophilic interactions [35–38]. To remove contributions of the ectodomains to dimerization, we generated chimeras consisting of the extracellular domain of RPTPα (EDα) and the transmembrane plus intracellular domain of LAR or RPTPμ and performed co-immunoprecipitations. EDα/LAR homodimers were detectable under control conditions, yet co-immunoprecipitation increased significantly in response to H_2O_2-treatment (Fig. 1D). Co-immunoprecipitation of chimeric EDα/RPTPμ was only detected after H_2O_2-treatment (Fig. 1D), similarly to RPTPα (Fig. 1C).

We have shown previously that the cytoplasmic domain of RPTPα is essential for the H_2O_2-induced change in dimerization state. H_2O_2 alters the conformation of RPTPα-D2, which is dependent on the catalytic site cysteine [8]. To investigate whether H_2O_2 induced changes in the conformation of other RPTPs as well, we performed limited tryptic proteolysis [39] on glutathione S-transferase (GST) fusion proteins consisting of the intracellular domains of RPTPα, RPTPμ, CD45 or LAR. The fusion proteins were digested with trypsin for 1, 3 or 5 min and run on SDS-PAGE gels (Fig. 2). Samples were treated with 1 mM H_2O_2 for 30 min, which predominantly results
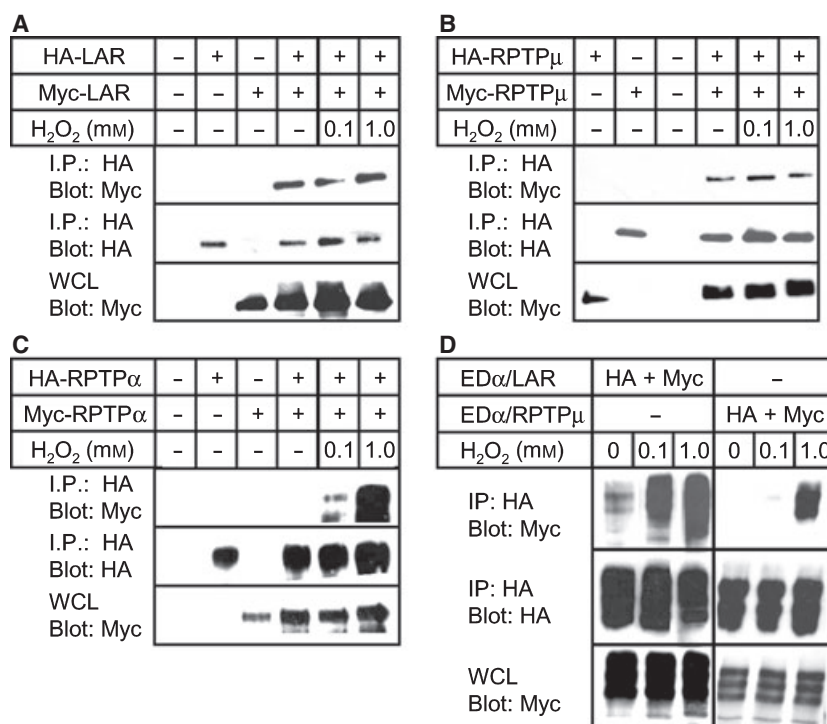
Fig. 1. Dimerization of LAR, RPTPμ and RPTPα. COS-1 cells were transiently co-transfected with (A) HA-tagged and/or Myc-tagged LAR, (B) HA-tagged and/or Myc-tagged RPTPμ or (C) HA-tagged and/or Myc-tagged RPTPα. Subsequently, cells were treated with 0.1 mM or 1 mM H_2O_2 for 5 min as indicated. HA-tagged proteins were immunoprecipitated using anti-HA IgG (12CA5), boiled in reducing Laemmli sample buffer, resolved on 7.5% SDS-PAGE gels and blotted. The blots were probed with anti-Myc antibody (9E10) and anti-HA IgG. Expression of the Myc-tagged constructs was monitored in the lysates (WCL). (D) COS-1 cells were co-transfected with HA- and Myc-tagged EDα/LAR or EDα/RPTPμ chimeras and treated with H_2O_2 for 5 min as indicated. HA-tagged proteins were immunoprecipitated, boiled in reducing Laemmli sample buffer, resolved on 7.5% SDS-PAGE gels and blotted. The blots were probed with anti-Myc IgG (9E10) and anti-HA IgG. Expression of the Myc-tagged constructs was monitored in the lysates (WCL).

in reversible oxidation [33] and limited proteolysis was repeated. The resulting protein bands were N-terminally sequenced by Edman degradation. Cleavage sites for RPTPα were found in the juxtamembrane region, in D1 and in D2 in the vicinity of the spacer region (Fig. 2A, supplementary Fig. S1). The difference in degradation pattern between reduced and oxidized RPTPα was striking. Novel and more intense bands (red arrows) were observed, as well as unchanged bands (black arrows) or decreased bands (green arrows) upon H_2O_2 treatment. This indicates that tryptic sites became more exposed following oxidation. Analysis of the cut sites in the 3D crystal structure of reduced RPTPα (data not shown) showed that all sites were positioned at the surface of the protein. As a control, GST-PTPα was incubated for 20 min with H_2O_2, which did not affect RPTPα at all (Fig. 2E). Pre-treatment of trypsin with 1 mM H_2O_2 for 20 min did not affect GST-RPTPα trypsinolysis (Fig. 2E), indicating that trypsin itself was not affected by H_2O_2.

Tryptic degradation of the other GST-PTP fusion proteins was also affected by oxidation (Fig. 2B–D). For GST-RPTPμ eight Coomassie-stainable bands were identified, five of which were affected by oxidation (Fig. 2B). The degradation pattern of CD45 showed a more complex digestion pattern and 14 bands were sequenced, which led to the identification of five tryptic sites. Interestingly, oxidation clearly induced changes in the tryptic digestion pattern (Fig. 2C), indicating that the conformation of CD45 changed upon oxidation. Tryptic digestion of LAR also showed a complex pattern with a striking difference between reduced and oxidized GST-LAR (Fig. 2D). The tryptic cleavage sites were localized throughout the cytoplasmic domains of these four RPTPs. One site was conserved in three of the four RPTPs at the −5 position relative to the TyrTrpPro-motif. However, in general the tryptic cleavage sites were not conserved (supplementary Fig. S1). Nevertheless, it is evident from this series of experiments that oxidation induced a conformational change in all four
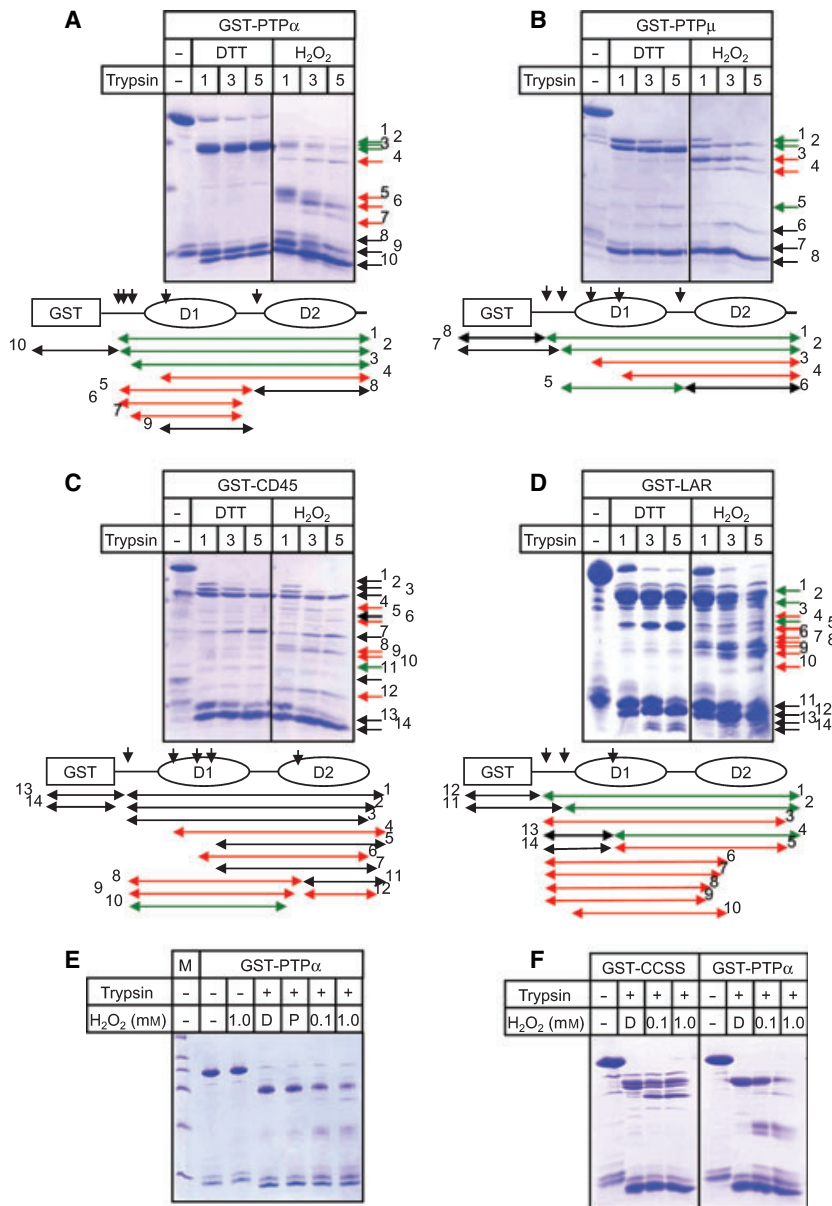
Fig. 2. Oxidation-induced conformational changes in the intracellular domains of RPTPs. GST-fusion proteins encoding the intracellular domain of (A) RPTPα, (B) RPTPμ, (C) CD45 and (D) LAR were cut for 1, 3 and 5 min with 5 μg·mL^{-1} trypsin under reducing conditions (10 mM dithiothreitol, DTT) or oxidizing conditions (1 mM H$_2$O$_2$; 20 min pre-treatment). Reactions were quenched by boiling for 5 min in reducing Laemmli sample buffer. Proteins were run on a 12.5% SDS-PAGE gel, blotted on PVDF membrane and stained with Coomassie. Bands of interest (shown by arrows) were cut out of the membrane and sequenced by Edman degradation. Black arrows indicate fragments that did not differ in intensity between reducing and oxidizing conditions. Green arrows indicate bands that were more intense under reducing conditions and red arrows indicate protein fragments that were more intense upon oxidation. Band numbers coincide with the numbers shown in the schematic representation of the protein fragments. (E) Trypsin itself is not affected by H$_2$O$_2$. GST-PTPα was treated with 1 mM H$_2$O$_2$ for 20 min by itself and run on SDS-PAGE gel. Trypsin was pre-treated with H$_2$O$_2$ for 20 min prior to proteolysis of GST-PTPα (P) for 1 min, or GST-PTPα was treated with 0.1 mM or 1 mM H$_2$O$_2$ for 20 min and digested with trypsin for 1 min as in (A). The fusion proteins were blotted on PVDF membrane and stained with Coomassie. (F) The catalytic cysteines of RPTPα are responsible for the oxidation-induced conformational change. GST-PTPα (wt) and GST-PTPαC433S/C723S were incubated with dithiothreitol (D) or with H$_2$O$_2$ as indicated and subsequently cut with 5 μg·mL^{-1} trypsin for 1 min. Membranes were stained with Coomassie blue.

RPTP cytoplasmic domains, resulting in a change in susceptibility to trypsin.

The dramatic change of the tryptic digestion patterns upon oxidation, led to the question as to what extent these differences were attributable to the catalytic cysteines. H$_2$O$_2$-treatment induced only minor changes in the limited tryptic degradation pattern of RPTPα-C433S/C723S in contrast to wild-type RPTPα. For instance, peptides 5, 6 and 7 (Fig. 2A) were induced by oxidation of wild-type RPTPα, but were not detected at all in the mutant (Fig. 2F). These data indicate that the observed change in degradation pattern in wild-type GST-RPTPα was the consequence of

oxidation of the catalytic site cysteines. Taken together, these limited tryptic proteolysis suggest that oxidation induced a change in conformation of the intracellular domain of this panel of RPTPs.

A functional consequence of the change in conformation in the cytoplasmic domain is a change in rotational coupling within RPTPα dimers [13]. We have developed an accessibility assay facilitating analysis of the conformation of full length RPTPα. In mutant RPTPα with a disulfide bond in the extracellular domain, the HA-tag to the N-terminal side of RPTPα is accessible or not accessible for the anti-HA-tag IgG, 12CA5, depending on the exact location of the
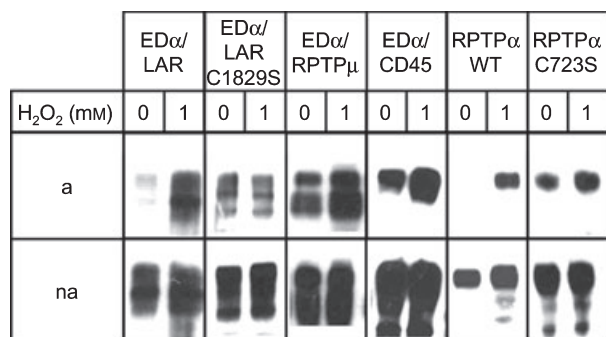
	EDα/LAR		EDα/LAR C1829S		EDα/RPTPμ		EDα/CD45		RPTPα WT		RPTPα C723S	
H₂O₂ (mM)	0	1	0	1	0	1	0	1	0	1	0	1
a												
na												

Fig. 3. Oxidation induced changes in rotational coupling of EDα/RPTP chimeras. COS-1 cells were transfected with the chimeras as indicated. Cells were treated with or without 1 mM H_2O_2 for 5 min and the accessible (a) and non-accessible (na) fractions of the proteins were obtained (see Material and methods). Samples were boiled in reducing Laemmli sample buffer, run on a 7.5% SDS-PAGE gel, blotted and immunostained with anti-HA IgG (12CA5). These experiments were repeated at least three times and representative blots are shown here.

disulfide bond. The epitope tag in wild-type RPTPα is not accessible under control conditions. The ectodomains of both monomers in the dimer are required for this effect, suggesting that the epitope tag of one monomer is masked by the ectodomain of the other monomer in the dimer, and vice versa. Since the epitope tag in monomeric RPTPα would be accessible, we concluded that dimerization of wild-type RPTPα under control conditions was extensive [13]. H_2O_2 induced a change in conformation, releasing the HA-tag which is now accessible for 12CA5. This phenomenon is dependent on the catalytic cysteine in RPTPα-D2 (Fig. 3). Here, we used the accessibility assay for the three EDα/RPTP chimeras in the presence and absence of H_2O_2. Basal level accessibility was detectable in all three chimeras. This may be due to subtle differences in quaternary structure of the chimeras compared to native RPTPα, or to the presence of low amounts of monomers. Nevertheless, there was a clear difference in accessibility between oxidized and reduced LAR, RPTPμ and CD45 chimeras, as was the case for RPTPα (Fig. 3). Mutation of Cys1829 in LAR-D2 abolished this effect, similarly to mutant RPTPα-C723S (Fig. 3). These results are consistent with oxidation inducing a change in rotational coupling, and suggest an important role for the catalytic cysteine of D2 in the process.

Discussion

Whether regulation of RPTPs by dimerization is a general feature is a subject of debate. There is ample evidence that RPTPs dimerize in living cells. Chemical cross-linkers show dimerization of CD45, RPTPα and Sap-1 [8,12,14,40]. In addition, we have used fluorescence resonance energy transfer to show homodimerization of RPTPα in living cells [7]. Dimerization of many RPTPs may be driven by their transmembrane domain, since the transmembrane domains of 18 out of 19 RPTPs mediated dimerization of fusion proteins above background levels [41]. The PTP domains are involved in homo- and heterodimerization as well [9–11]. Co-immunoprecipitation experiments demonstrate dimerization of full length RPTPα [8], CD45 [15], RPTPε [42] and RPTPσ [43]. LAR and RPTPμ also dimerized constitutively (Fig. 1).

Structural and functional evidence supports the hypothesis of an important role for dimerization as a regulator of RPTPs [6,12,16]. All RPTP crystal structures solved to date contain wedge-like structures to the N-terminal side of the D1, similar to the inhibitory wedge in RPTPα. However, the crystal structures of the intracellular domains of LAR [19] and CD45 [20] suggest that dimerization is unlikely to occur due to steric hindrance, assuming that there is no flexibility in the cytoplasmic domain of RPTPs. Using limited tryptic proteolysis, we found differences in the patterns of RPTPα, RPTPμ, LAR and CD45 before and after H_2O_2-treatment (Fig. 2), demonstrating that oxidation induced changes in the conformation of the cytoplasmic domains of these RPTPs. These results suggest there is flexibility in the cytoplasmic domain of RPTPs, and are evidence against rigid conformations that prohibit regulation of dimerized RPTPs.

Oxidation induced a conformational change in the cytoplasmic domain of RPTPα, RPTPμ, LAR and CD45 (Fig. 2) and concomitant changes in rotational coupling (Fig. 3). The catalytic cysteine in D2 of RPTPα and LAR was required for the change in rotational coupling. Oxidation-induced changes in rotational coupling may drive RPTP dimers into an inactive conformation, similarly to RPTPα [8]. Alternatively, changes in rotational coupling may result in binding to different ligands extracellularly, which would represent 'inside-out' signaling [13]. This model is supported by the finding that only dimeric RPTPσ ectodomain bound ligand, and that changes in rotational coupling within the RPTPσ ectodomain affect ligand binding [43]. Our results suggest that oxidation-induced changes in the cytoplasmic domain may result in binding to different ligands extracellularly, and hence suggest that oxidation may regulate 'inside-out' signaling.

We demonstrate here that RPTPs can be regulated by oxidation using H_2O_2 at physiologically relevant concentrations (0.1–1.0 mM). Growth factor receptor

activation results in the production of ROS in cells, equivalent to the exogenous addition of upto 2 mM H_2O_2 [24]. This prompted us to test whether growth factor receptor activation induced co-immunoprecipitation of RPTPα and/or changes in accessibility. Unfortunately, to date we have not yet identified growth factors or other stimuli that induced differences in co-immunoprecipitation of RPTPα. We hypothesize that this is due to localized production of ROS at sites where RPTPα is not localized. We will continue to search for stimuli that regulate oxidation of RPTPs.

In conclusion, the results we present here are consistent with dimerization being a general regulatory mechanism for RPTPs. We provide evidence that RPTPs dimerize constitutively. Moreover, oxidation induced conformational changes in the cytoplasmic domain of all four RPTPs tested, altering rotational coupling within RPTP dimers. These conformational changes may regulate the catalytic activity or function of RPTP dimers.

Materials and methods

Constructs

HA- and Myc-tagged PSG5-13 eukaryotic expression vectors were made containing full-length RPTPμ or LAR. PSG5-13 vectors containing tagged RPTPα were previously described [8]. Chimeras encoded the HA- or Myc-tagged extracellular domain of RPTPα (1–141), together with the transmembrane region and the intracellular domain of LAR (1235–stop), CD45 (426–stop) or RPTPμ (865–stop). Mutants were made by site directed mutagenesis. pGEX-based expression vectors encoding GST fusion proteins contained RPTPμ (865–1452), Lar (1275–1897) or CD45 (448–1152).

Cell Culture, immunoprecipitation and immunoblotting

COS-1 cells were 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. The next day, COS-1 cells were serum starved and 16 h later the cells were treated with variable concentrations of H_2O_2 for 5 min or left untreated.

COS-1 cell lysis was done by scraping in cell lysis buffer (CLB; 50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM aprotinin, 1 mM leupeptin, 1 mM ortho-vanadate). Cell lysates were cleared and an aliquot was boiled in equal volume 2× Laemmli sample buffer and run on a 7.5% SDS-PAGE gel.

Immunoprecipitation was done with anti-HA IgG 12CA5 and protein A sepharose for 2–3 h at 4 °C. Following immunoprecipitation, beads were washed four times with HNTG (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100) and subsequently boiled in Laemmli sample buffer for 5 min and run on an SDS-PAGE gel. Proteins were subsequently transferred by semi-dry blotting to a poly(vinylidene difluoride) (PVDF) membrane. Immunoblotting was visualized by enhanced chemoluminescence.

Accessibility assays were performed as previously described [13]. Transfected COS-1 cells were treated with or without H_2O_2 for 5 min and were incubated on ice with anti-HA IgG for 1 h. After washing, cells were lysed in CLB and lysates were incubated with protein A sepharose beads for 30 min to collect the accessible (a) fraction of the protein. The lysates were removed and anti-HA tag immunoprecipitations were done on these lysates to collect the non-accessible (na) fraction. All immunoprecipitates were washed 4× with HNTG and samples were loaded on 7.5% SDS-PAGE gel for immunoblotting.

Limited tryptic proteolysis and Edman degradation

GST-fusion proteins were incubated with 1 mM H_2O_2 or with 10 mM dithiothreitol for 20 min and cut with 5 μg·mL^{-1} trypsin for 1, 3 or 5 min. Reactions were quenched by boiling in 2× Laemmli sample buffer for 5 min. Proteins were loaded on a 12.5% SDS-PAGE gel and blotted. The blots were stained with Coomassie blue and protein bands of interest were cut out and sequenced by Edman degradation at Department of Lipid Chemistry, Utrecht University.

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Supplementary material

The following supplementary material is available online:

Fig. S1. Mapping of the tryptic cleavage sites.

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