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Forum Review

Functions and Mechanisms of Redox Regulation of Cysteine-Based Phosphatases

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ABSTRACT

Reactive oxygen species (ROS) have been implicated as mediators of cell-signaling responses, particularly in pathways involving protein tyrosine phosphorylation. One mechanism by which ROS are thought to exert their effects is through the reversible regulation of cysteine-based phosphatases (CBPs). The CBPs, which include protein tyrosine phosphatases (PTPs), dual-specificity phosphatases, low-molecular-weight PTPs, and the lipid phosphatase PTEN, all contain a nucleophilic catalytic cysteine within a conserved motif that enables these enzymes to dephosphorylate phosphoproteins or phospholipids. In addition to enabling phosphatase activity, the nucleophilic catalytic cysteines of CBPs are also highly susceptible to oxidation, a property that permits redox regulation of this enzyme family. In this review, we discuss the evidence implicating ROS as mediators of CBP activity within signaling pathways and discuss how specificity of ROS-dependent signaling involving CBPs may be achieved. We also discuss the molecular mechanisms that facilitate the stabilization of a reversibly oxidized form of the catalytic cysteine. These mechanisms include the formation of disulfide bonds or the formation of a sulfenamide bond, a novel mechanism that was identified for PTP1B. Formation of either type of covalent bond may be accompanied by dramatic structural rearrangements that can affect downstream signaling events and allow for multitiered enzyme regulation. *Antioxid. Redox Signal.* 7, 560–577.

INTRODUCTION TO CYSTEINE-BASED PHOSPHATASES (CBPS) AND REACTIVE OXYGEN SPECIES (ROS)

The first CBP to be characterized was the protein tyrosine phosphatase 1B (PTP1B) that specifically dephosphorylates phosphotyrosine (pTyr) residues. Since the characterization of PTP1B in the late 1980s, the family of CBPs has expanded to ~100 members (see Table 1) that function in diverse signaling pathways relevant to nearly all aspects of cell life (for reviews, see 1, 2, 6, 34, 85). The CBPs share the common function of hydrolyzing phospho-ester bonds in proteins and/or lipids via a conserved cysteine-based mechanism. The shared structural and catalytic feature of these enzymes is a $Cys(Xaa)_5Arg$ sequence motif (where Xaa is any amino acid), termed the protein tyrosine phosphatase (PTP) signature motif. This simple motif contributes most of the structural features required for phosphate recognition and phospho-ester hydrolysis.

There is evidence that CBPs can be differentially regulated by phosphorylation, intra- and intermolecular interactions, alternative splicing, transcription, and translation (2, 34), but recently it has become apparent that this family of enzymes may be regulated by a common mechanism, *i.e.,* reversible oxidation of the catalytic cysteine. In this review, we will discuss the biochemical, structural, and biological evidence suggesting that CBPs are regulated by redox mechanisms. We will explain the mechanisms of redox regulation known for specific CBPs and discuss the current state of knowledge and challenges for understanding how redox regulation of CBPs may function *in vivo*.

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TABLE 1. PROPERTIES OF CBPS

CBP family	*Substrates*	*$C(X)_5R$ motif*	*Number of human genes**	*Examples*	*Redox regulation*	*Comments*
PTPs Tyrosine-specific	pTyr	HCSXGXGR(S/T)G	38	Cytosolic: PTP1B, SHP-2 Receptor-like: CD45, LAR, RPTPα	Cyclic sulfenamide (PTP1B)	PTP1B founding member of class
DSPs Dual specificity	pTyr or pSer/pThr or phosphoinositides	$HC(X)_5R(S/T)G$	61	KAP, Cdc14, VHR, MKPs, PTEN, myotubularin	Disulfide (PTEN); stable SOH (VHR)	Subfamily of PTPs, protein and lipid phosphatases
LMW-PTPs Tyrosine-specific	pTyr	$C(X)_5R(S/T)$	1	LMW-PTP	Disulfide	Fold similar to *S. aureus* arsenate reductase
Cdc25 Cell cycle phosphatase of CDKs	pTyr or pSer/pThr	$HC(X)_5R$	3	Cdc25A, Cdc25B, Cdc25C	Disulfide	Fold similar to rhodonase

*Data were obtained from Alonso *et al.* (1).

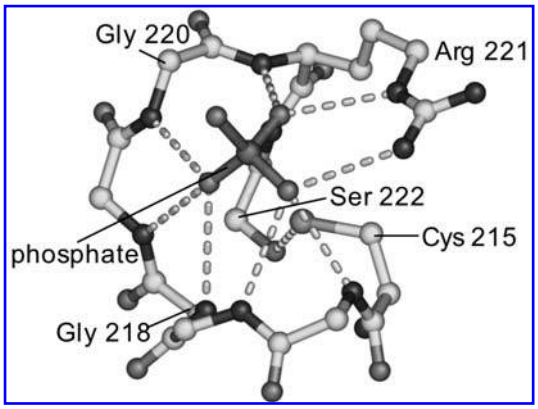

FIG. 1. Structure of the conserved PTP loop in CBPs created by the conserved Cys(Xaa)$_5$Arg motif. Shown is the PTP loop of PTP1B (residues Cys215 to Ser 222) with a bound phosphate ion. Main chain amides and the guanidinium side chain of Arg221 coordinate the phosphate, positioning it adjacent to the nucleophilic Sγ-atom of Cys215. The positive electrostatic potential promotes the low pK_a of the catalytic Cys residue. The side chains of residues Ser216, Ala217, and Ile219 have been omitted for clarity. Figures were generated using PYMOL (http://www.pymol.org).

Redox regulation of proteins, in general, occurs primarily when cysteine residues within the protein, or bound transition metals, react with ROS, such as hydrogen peroxide (H_2O_2) or superoxide ($O_2^{\bullet -}$), or with reactive nitrogen species, such as nitric oxide (NO), *S*-nitrosothiols (RSNO), or peroxynitrite (ONOOH). Reactions of cysteines with mild oxidizing or nitrosylating reagents, such as low concentrations of H_2O_2 or NO, can lead to reversible modulations of protein activity. Reactions with strong oxidizing agents, such as hypochlorous acid (HOCl), singlet oxygen ($^1O_2$), or hydroxyl radical ($OH^{\bullet}$) or high concentrations of H_2O_2 or ONOOH, however, can lead to an extreme form of redox regulation in which enzymes become permanently modified, ultimately inducing apoptosis and cell death.

In the case of the CBPs, redox regulation can occur at the conserved catalytic cysteine that exists within the Cys-Xaa$_5$-Arg PTP signature motif (for a recent review, see 17). The architecture of the catalytic sites of CBPs creates an acidic environment around the catalytic cysteine that lowers the p*K*a of these residues to ~5.0–6.7 in comparison with ~8.0 for free cysteine (Fig. 1) (23, 25, 48, 95). Thus, at physiological pH, the catalytic cysteines of CBPs are deprotonated. This property enables them to act as nucleophiles in the first step of catalysis (see *Introduction to CBPs* below), but also makes them highly susceptible to reactions with ROS, and, to a lesser degree, with reactive nitrogen species. Oxidation or nitrosylation of the catalytic cysteine renders the residue unable to act as a nucleophile, and the enzyme loses its phosphatase activity. Although there is evidence that PTPs can become inactivated by reactions with RSNO and ONOOH, it is not clear that the catalytic cysteine becomes nitrosylated (46, 82). Thus, the remainder of this review will focus on modifications of CBPs by ROS, and the role of these redox-regulatory mechanisms within cell signaling pathways.

ROS AND CELL SIGNALING

Historically, reactions of proteins with ROS have been associated primarily with a variety of cytotoxic effects, such as DNA damage, transformation, and neurodegenerative diseases. More recently, it has become clear that lower concentrations of ROS can function beneficially as regulatory molecules in cell signaling pathways (for reviews, see 28, 29, 68). For example, a large number of cellular stimuli, including hormones, growth factors, cytokines, and agonists of heterotrimeric G protein-coupled receptors, promote an increase in cellular levels of ROS (3, 40, 72, 79, 92). Blocking the accumulation of ROS by means of antioxidants or enzymes that break down H_2O_2 actually prevents optimal activation of several signaling pathways (3, 51, 79). Furthermore, addition of exogenous H_2O_2 to cells can mimic the effects of growth factors or hormones and lead to hyperphosphorylation of receptor tyrosine kinases (RTKs) (39,79). These observations led to the suggestion that PTPs, because of their ability to dephosphorylate RTKs and their high susceptibility to inactivation by oxidation at physiological pH, are targets of ligand-induced ROS generation (79). This would imply that complete activation of tyrosine kinase signaling cascades requires both the inhibition of PTPs by oxidation and the activation of RTKs by phosphorylation (Fig. 2).

The suggestion that PTPs and other related CBPs can be regulated by ligand-stimulated ROS generation has now been supported by experimental evidence (see *Oxidation of CBPs* below). Other families of proteins, including transcription factors, small GTPases, kinases, and serine threonine phosphatases, have also been shown to be affected by ROS either

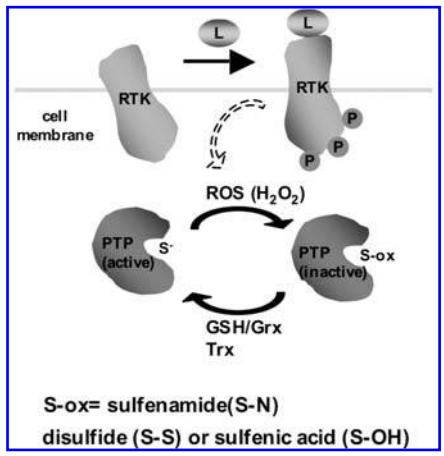

FIG. 2. Schematic for the oxidation of PTPs within cell signaling pathways. Starting from the left, when ligands bind to RTKs, they promote the generation of ROS, most likely via either NADPH oxidases or the metabolism of AA. The ROS that is produced, usually H_2O_2, then inhibits the PTPs by oxidizing the catalytic cysteine. This inhibition of phosphatase activity enables the complete phosphorylation of the RTK and the full activation of downstream signaling pathways. Reducing enzymes can then return the PTPs to their active state, resulting in the down-regulation of the signaling pathways. Grx, glutaredoxin; Trx, thioredoxin.

in response to physiological stimuli or under conditions of oxidative stress (68). However, the diverse and ubiquitous nature of ROS and cellular thiols, such as glutathione (GSH), and the complexity of the redox environment within cells have made it challenging to assess the detailed mechanisms by which ROS participate in controlled regulatory pathways. Many questions remain regarding the roles of ROS in cell signaling. For example, to what degree is a small, readily diffusible molecule such as H_2O_2 able to induce specific signaling responses? Which proteins are involved in ROS production in response to particular cell stimuli? And how do cells differentiate between toxic and beneficial levels of ROS?

It is likely that answering these questions will require a detailed knowledge of the cellular environment in which the redox signaling occurs (54, 62, 84). In a healthy cell, the reduction potential [determined using the Nernst equation from the ratio of oxidized to reduced species (69)] varies from $E° = -280$ mV in the mitochondria, the most reducing organelle, to $E° = -180$ mV in the endoplasmic reticulum, and ranges from -230 mV to -260 mV within the cytoplasm. This "redox gradient" alone could affect the ability of ROS to oxidize a protein in a given subcellular location, but the likelihood that ROS will oxidize a signaling protein will also depend on the proximity of the signaling protein to the site of ROS production, and on the local concentration of proteins that break down ROS such as superoxide dismutase, catalase, or peroxiredoxins. When ROS do react with a signaling molecule, the length of time that the protein will be inactivated will also depend on the proximity to enzymes, such as glutaredoxin and thioredoxin, that catalyze thiol reduction. For these reasons, to understand redox regulation of CBPs it will be important to ask where ROS originate from within a cell, how they are produced in signaling pathways, and how their effects would be reversed. These will be covered briefly in the next two sections.

Sources of ROS

There are at least three known sources of ROS within cells: the mitochondrial electron transport chain, NADPH oxidases, and the lipoxygenase- or cyclooxygenase-catalyzed reaction that converts arachidonic acid (AA) to prostaglandins and leukotrienes (62). It is estimated that 1–2% of the molecular oxygen that is consumed in the mitochondria is converted first to superoxide by a ubiquinone-catalyzed one-electron reduction and then to H_2O_2 by manganese-dependent superoxide dismutases (15). ROS from mitochondrial sources make up the majority of the ROS in the cell, and there is now evidence that these ROS are not just by-products of metabolism, but that they have an important roll in cell signaling pathways. For example, ROS produced in the mitochondria have been implicated in cellular responses to hypoxia, glutamate, serum deprivation, and integrins (62, 89).

Other cellular stimuli, such as growth factors, hormones, and cytokines, are thought to produce ROS via either NADPH oxidases or the oxidative metabolism of AA (see Fig. 2) (64, 80, 90). Production of ROS in response to platelet-derived growth factor (PDGF), epidermal growth factor (EGF), tumor necrosis factor-α, interleukin-1β, and possibly other cellular stimuli is dependent on Rac1 activation, a step that is required for both activation of NADPH oxidases and the production of AA (80). Inhibitors of NADPH oxidases or lipoxygenase also prevent the production of ROS in response to various stimuli (50, 54, 61, 90). It appears that certain cellular stimuli may induce ROS production via AA metabolism, whereas others utilize NADPH oxidases. There are several examples where only lipoxygenase inhibitors and not NADPH oxidase inhibitors prevent ROS production (61, 90).

In its activated state, the prototypic NAPDH oxidase from phagocytes is a protein complex of six subunits, including the activator protein Rac (74). The catalytic subunit gp91phox (or Nox2) contains C-terminal homology to flavoprotein dehydrogenases and an NADPH binding site. Recent cloning studies have identified a small family of Nox enzymes that are homologues of gp91phox and are expressed in a variety of nonphagocytic cell types (16). Overexpression of members of this protein family causes elevated levels of ROS and is a characteristic of several types of cancer cells (41). Other types of studies have also linked ROS to tumorigenesis; for instance, many cancer cells overproduce ROS (81), and increased ROS levels in Ras-transformed fibroblasts correlate with increased mitogenesis (33). Recent studies showed that overexpression of Nox4, a component of NADPH oxidases in some nonphagocytic cell types, could compensate for effects caused by overexpression of PTP1B, suggesting that Nox4 is directly involved in the production of ROS that, in turn, inhibit PTP1B in response to insulin (discussed below) (52).

Removal of ROS or reversal of their effects

The localization of enzymes that destroy ROS directly or reduce modified cysteines and the concentrations of small molecule antioxidants such as GSH are also likely to be important components of overall ROS signaling. This is especially true because ROS are small molecules and would diffuse rapidly across a cell in the absence of antioxidants. Hence, the localization of antioxidant enzymes could significantly affect the ability of ROS to operate in cell signaling pathways. There are several families of enzymes, *e.g.,* catalases, glutathione peroxidases, and peroxiredoxins, that break down H_2O_2 directly. There are also families of enzymes, the glutaredoxins and thioredoxins, that can catalyze the reduction of cysteine residues that have been modified by oxidation. Until recently, it was believed that the only types of oxidative modifications of cysteines that could be reversed were the formation of sulfenic acids (Cys-SOH) and disulfide bonds. We now have evidence, however, that cysteines can also be reversibly modified by the formation of sulfenamide bonds within proteins in which the sulfur atom of the cysteine is covalently linked to the neighboring amide nitrogen (see *The Sulfenamide Bond* below). Furthermore, two studies have revealed the potential ability of enzymes in cells to catalyze the reduction of a higher oxidation species of cysteine, sulfinic acid ($Cys\text{-}SO_2$). Woo *et al.* described an enzyme present in mammalian cells that can reduce a sulfinic acid at the catalytic site of the peroxiredoxins back to a thiol (91). More recently, Biteau *et al.* have reported the identification of a protein, termed sulfiredoxin, in yeast that is conserved in higher eukaryotes and catalyzes the reduction of sulfinic

acids of peroxiredoxins in an ATP- and Mn^{2+}/Mg^{2+}-dependent manner (9).

CYSTEINE-BASED PHOSPHATASES

Introduction to CBPs

The CBPs are broadly classified into three topological families: the conventional (high-molecular-weight) PTPs, the Cdc25 phosphatases, and the low-molecular-weight PTPs (LMW-PTPs) (Table 1). The conventional PTPs include the tyrosine-specific PTPs, the dual-specificity PTPs (DSPs) that dephosphorylate serine and threonine as well as tyrosine, and the lipid phosphatase PTEN. Cdc25 phosphatases are also DSPs in function, but have a rhodanese-like fold for their catalytic domains (27). The LMW-PTPs are tyrosine-specific. Other than the Cys-Xaa_5-Arg PTP signature motif, there are few sequence similarities between the three CBP families (5, 26). Although they possess different topologies and therefore are unlikely to have evolved from a common ancestor, the three-dimensional structures of the catalytic domains of the three CBP protein families are strikingly similar. They all possess an overall structure with a core catalytic domain composed of four parallel β-strands surrounded on both sides by α-helices (Fig. 3). The $Cys(Xaa)_5Arg$ motif creates a very similar structural motif, termed the PTP loop, connecting a central β-strand to an α-helix, at the center of the catalytic site. Unlike the catalytic domains, the regulatory domains of the CBPs vary significantly even within the highly conserved PTP family; thus, much of the diversity within the families is conferred by the regulatory domains.

The dephosphorylation reaction catalyzed by the three families of CBPs is essentially identical (Fig. 4). It is a two-step process involving the formation of a cysteinyl-phosphate intermediate followed by water-mediated hydrolysis to release inorganic phosphate. In the case of the tyrosine-specific PTPs, the catalysis involves four conserved loop regions of the protein (Fig. 3). In the first step of catalysis, the pTyr substrate is positioned in a groove on the surface of the protein (35). The catalytic cysteine is situated at the base of the groove within the PTP loop, the region of the protein that contains the PTP signature motif (Figs. 1 and 3) (5). The depth of the groove is defined by a second region, the pTyr loop, which helps define the specificity of these proteins for pTyr substrates (35). Substrate engages this positively charged pocket, so that the catalytic cysteine is positioned in such a way that it can act as a nucleophile toward the pTyr substrate. A third loop region in tyrosine-specific PTPs known as the WPD loop closes over the substrate, enabling an

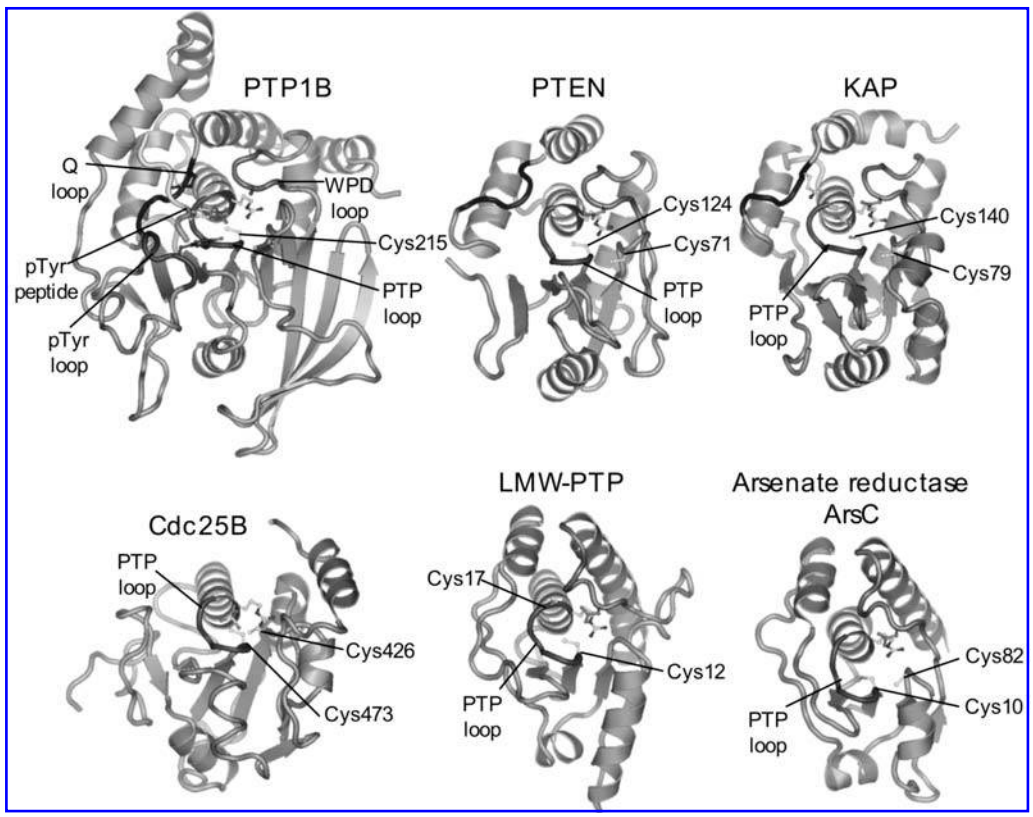

FIG. 3. The structures of the catalytic domains of CBPs. Shown are PTP1B (70) (a tyrosine-specific PTP), KAP (76) and PTEN (42) (DSPs), the catalytic domain of Cdc25 (67), LMW-PTP (77), and arsenate reductase (ArsC) (93). All enzymes are in their reduced states, *i.e.,* the catalytic Cys is present as a thiolate anion, or has been substituted by Ser (PTP1B, KAP, ArsC).

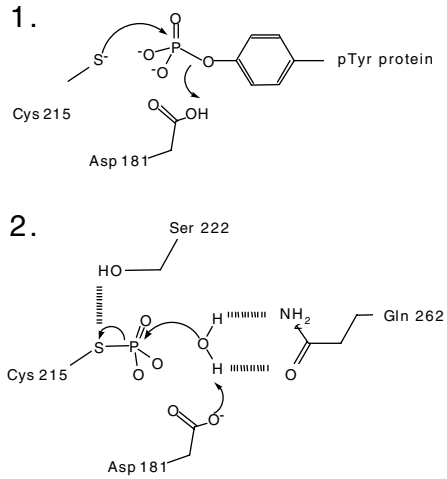

FIG. 4. The two-step catalytic mechanism of the PTPs. The residue numbering refers to PTP1B.

invariant Asp residue to act as a general acid helping to form a cysteinyl phosphate intermediate (35). In the second step of catalysis, the cysteinyl phosphate intermediate is hydrolyzed by a water molecule coordinated by a glutamine residue in the fourth catalytic loop in PTPs, the Q loop (63). In this second step of the catalytic mechanism, the WPD loop acts as a base. The DSPs, LMW-PTPs, and Cdc25-phosphatases also utilize an acid-mediated hydrolysis mechanism, but there is evidence that Cdc25 may utilize a different mechanism for substrates with a low leaving group pK_a (25, 53).

Oxidation of CBPs

Even before the discovery of tyrosine (de)phosphorylation, early work by Czech *et al.* demonstrated that a component of the insulin signaling pathway leading to glucose transport was sensitive to sulfhydryl oxidation (21). They showed that the reducing agent *N*-methylmaleimide could block the effects of H_2O_2 and insulin on glucose transport (21). Based on the work of Czech and others who demonstrated that exposure of cells to H_2O_2 leads to enhanced phosphorylation of the insulin receptor (39), Heffetz *et al.* proposed that phosphatase inhibition may be involved in the insulinomimetic effects of H_2O_2 (32). They conducted phosphatase activity assays on cells stimulated with H_2O_2 and noted a 50% loss in phosphatase activity upon exposure to oxidants. The phosphatase activity from cytosolic extracts of rat hepatoma cells exposed to H_2O_2 was also found to be inhibited after exposure to H_2O_2 (31). Sullivan *et al.* demonstrated that the inhibition of phosphatases by H_2O_2 was reversible. Exposure of Her2 cells to H_2O_2 led to a 40% reduction in phosphatase activity, and this activity could be recovered completely within 60 min (78). Cyclohexamide, a protein synthesis inhibitor, had no effect on the recovery of PTP activity in Her2 cells, implying that the recovery of PTP activity after oxidation was the result of reduction of the thiols, and not due to *de novo* PTP synthesis. Investigation into how UVC, UVB, UVA, H_2O_2, and iodoacetamide (IA) affected tyrosine kinase signaling also led to the conclusion that the oxidation of sensitive thiols of PTPs are affected by oxidizing agents (38). Now, it is widely accepted that PTPs, and the larger family of CBPs, are sensitive to oxidation, and a number of studies have revealed the mechanism of action of ROS for various CBPs. These will be reviewed in the following section.

Cytosolic tyrosine-specific PTPs

PTP1B. PTP1B is the prototypic member of the PTP family, and functional and structural studies of this protein have provided insights into the entire family. A variety of studies have demonstrated that the generation of ROS in response to growth factor stimulation of cells is an important mechanism for control of PTP1B activity *in vivo* and the activation of receptor-mediated signaling. Lee and colleagues reported that PTP1B becomes oxidized in A431 human epidermoid carcinoma cells in response to EGF stimulation (43). They established that iodoacetic acid would react with the catalytic cysteine of reduced PTP1B, but not with oxidized PTP1B. The amount of iodoacetic acid incorporation into cell lysates could then be utilized as a measure of the extent to which PTPs had become oxidized. Iodoacetic acid-labeled PTP1B immunoprecipitates from cells stimulated with EGF for 10 min contained 27% less radioactivity than immunoprecipitates from unstimulated cells. The reduction in iodoacetic acid incorporation was maximal at 10 min of EGF stimulation and returned to basal levels after 40 min of EGF stimulation. Lee *et al*. noted that the oxidation of PTP1B in these experiments was partially reversible and that PTP1B could be reactivated more effectively by thioredoxin than glutaredoxin (43).

Insulin stimulation of hepatoma and adipose-like cells also leads to a reduction in overall PTP activity, and specifically PTP1B activity (51). This is particularly significant because numerous studies have implicated PTP1B as a negative regulator of insulin signaling that dephosphorylates stimulatory pTyr residues of the insulin receptor kinase activation segment (24, 37, 70). A number of recent studies strongly implicate H_2O_2 as the mediator of PTP1B inactivation, providing a mechanistic explanation for the insulinomimetic effects of H_2O_2 discovered by Czech and others some 30 years ago (21, 39). When assayed under anaerobic conditions, the overall PTP activity in HepG2 and 3T3-L1 adipocytes dropped significantly after exposure to insulin (51). These effects were reversed when the cell lysates were incubated with dithiothreitol (DTT). Immunoprecipitated PTP1B, 2 min after insulin stimulation, was reduced to 12% of control values. However, preincubating lysates with DTT prior to the activity assay restored activity to 72%, indicating that inactivation of PTP1B was due to reversible oxidation. In support of this notion, preincubating the cells with catalase, an enzyme that specifically reacts with H_2O_2 breaking it down to water and molecular oxygen, prevented PTP1B inactivation, indicating that H_2O_2 is responsible for this loss of activity. Catalase also affected the tyrosine autophosphorylation of the insulin receptor (IR) that occurs in response to insulin. Addition of catalase to cells did not affect the basal level of IR phosphorylation, but reduced the insulin-stimulated phosphorylation of the IR by ~40%.

As mentioned above, an important question to resolve is if, and how, growth factor stimulation of cells can specifically inhibit particular PTPs. It is therefore of interest that EGF and insulin stimulation of cells both inhibit only a portion of the PTP1B and total phosphatase activity. In both examples, exposure of cells to H_2O_2, which would function as a nonspecific oxidant, inhibited higher percentages of PTP activity than the physiological stimuli, suggesting that the growth factor-induced ROS may target a specialized pool of PTPs (43, 51).

SHP-2 and in-gel phosphatase assays. Studies of PDGF signaling using an in-gel phosphatase assay have provided further evidence that ligand-induced ROS inactivates a specialized pool of PTPs (54). Nonstimulated, PDGF-stimulated, or H_2O_2-stimulated Rat1 fibroblasts were lysed and exposed to IA at various time points. Proteins in the lysates were separated by one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and subject to in-gel phosphatase assays. For the assay, the proteins on the gel were denatured and subsequently renatured in the presence of DTT (13). Following renaturation, only the nonalkylated, reversibly oxidized PTPs that could be refolded (this assay is not suitable for detecting reversibly oxidized receptor PTPs because they cannot be refolded) could recover their activity. Thus, only PTPs that were oxidized during IA labeling could be visualized on a gel using ^{32}P-labeled Tyr phosphorylated protein as a substrate. In the cells exposed to H_2O_2, multiple PTPs had become reversibly oxidized. PDGF stimulation of cells, however, resulted only in the reversible oxidation of one PTP, SHP-2. Similar concentrations of EGF and fibroblast growth factor at the time point for which ROS production was measured did not result in the production of ROS in Rat1 fibroblasts nor in the reversible inactivation of SHP-2, suggesting that the SHP-2 inhibition was specific to PDGF.

Meng *et al.* also demonstrated that it is the SHP-2 that is recruited to PDGF receptors that becomes oxidized upon PDGF stimulation (54). They constructed chimeric receptors of the PDGF intracellular and juxtamembrane domains with the extracellular domain of human granulocyte colony stimulating factor (G-CSF) receptor. As G-CSF is not expressed in Rat1 fibroblasts, they could then compare the wild-type PDGF cytoplasmic domain with a mutant (Tyr1009) in which the SHP-2 docking sites were disrupted. When the cells were stimulated with G-CSF, oxidation of SHP-2 only occurred in the cells containing the chimeric receptors with the wild-type PDGF cytoplasmic domain, and not in the cells with the mutant. This implied that it is the SHP-2 that is recruited to the PDGF that becomes oxidized, an implication that is consistent with the hypothesis that ROS produced in response to growth factors exerts effects locally.

Pani *et al.* have suggested that EGF stimulation of cells also affects the redox state of SHP-2 (61). They used the SHP-2 phosphorylation state as an indirect readout of SHP-2 activity and showed that, in immunoprecipitates with an anti-SHP-2 antibody, the EGF receptor is less phosphorylated in dense cells than in sparse cells. Using an oxidizable fluorescein derivative, they also noted that the production of ROS in fibroblast cells in response to EGF is cell density-dependent and proposed a model that impaired production of ROS and an associated increase in PTP activity in confluent cells are important components in mediating contact inhibition.

Receptor PTPs (RPTPs)

RPTPα/leukocyte common antigen-related phosphatase (LAR). RPTPs consist of extracellular domains and two intracellular PTP domains. The membrane proximal D1 domains of RPTPs are catalytically active similar to the catalytic domains of cytosolic PTPs. The D2 domains, which are distal to the membrane, are catalytically inactive despite having a conserved PTP motif sequence. Substitutions of the Asp residue of the WPD loop, and the Tyr residue in the pTyr loop (which are required for PTP activity), reduce or abolish the catalytic activity of D1 domains (88). Significantly, mutation of these residues to the Asp and Tyr residues found in catalytically active PTPs confers catalytic activity upon the RPTP D2 domains (12, 47, 59). These observations have raised questions concerning the function of the D2 domains, and specifically why the PTP motif, containing a catalytically active Cys thiolate residue, is conserved when it seems unlikely that any D2 domains function as protein phosphatases.

It has now been demonstrated that the conserved Cys residue in the PTP motif of certain RPTP D2 domains is sensitive to oxidation, suggesting that D2s can act as redox sensors to regulate RPTP activity. Using fluorescence resonance energy transfer between cyan fluorescent protein and yellow fluorescent protein fused to the N- and C-termini, respectively, of the spacer region plus the D2 domain of PTPα, Blanchetot *et al.* demonstrated that oxidation of Cys723 within the D2 domain of PTPα (the equivalent position as the catalytic cysteine in active PTP domains) causes a conformational change in the D2 domain (10). They also showed that oxidation of Cys723 stabilizes existing dimers of full-length PTPα. Mutation at Cys433, the catalytic cysteine of the D1 domain, did not have any effect on dimer stabilization. Using similar methods, Blanchetot *et al.* also demonstrated the formation of heterodimers between PTPα and another RPTP, LAR, in response to oxidative stress (11). Subsequently, oxidation of the D2 domain of RPTPα has also been shown to affect the conformation of the extracellular domains of RPTPα, raising the interesting possibility that RPTPα is capable of inside-out signaling (86).

Oxidation of the RPTPs has also been investigated using a generic antibody-based approach for detection of oxidized PTPs (65). The method is based on the recognition of a sulfonic acid-modified PTP loop containing the VHCSAG sequence motif, characteristic of tyrosine-specific PTPs. An interesting result from this study was the observation that the RPTPα D2 domain is significantly more sensitive to oxidation than its D1 domain. When cells were exposed to either H_2O_2 or UV irradiation, oxidation of the RPTPα D2 domain was detected, with little or no oxidation of the D1 domain. Using the antibody detection method, Persson *et al.* (65) also confirmed the results of Meng *et al.* (54) that SHP-2 becomes oxidized in response to PDGF stimulation. The antibodies present a novel method for detection of oxidized PTPs *in vivo,* which overcomes some of the problems inherent with the in-gel phosphatase assay.

LMW-PTPs. LMW-PTPs have little sequence similarity to other subfamilies of tyrosine phosphatases, except for the CX_5R catalytic site motif (94). The catalytic cysteines of both isozymes of human LMW-PTPs are sensitive to oxidation by H_2O_2 and can form a disulfide bond with a vicinal cysteine (14). *In vivo* LMW-PTP is rapidly and reversibly oxidized to the disulfide-bonded state in response to PDGF stimulation, thereby inhibiting the phosphatase (18). Chiarugi *et al.* monitored the level of LMW-PTP oxidation using an IA-fluorescein labeling and immunodetection method, and showed that LMW-PTP oxidation was transient, reaching a maximum level of 80% inactivation 10 min after PDGF stimulation, but recovering to 70% of the prestimulus activity after 40 min (18). Reactivation was most likely via a GSH-mediated reduction of the catalytic site disulfide.

Recently, Nimnual *et al.* demonstrated that the inhibition of LMW-PTP by ROS is required for Rac-induced cell spreading and lamellipodia formation (60). They showed that production of ROS by the small GTPase Rac, a protein that promotes cell spreading and migration, leads to the down-regulation of Rho, another small GTPase that promotes cell contractility and adhesion. The down-regulation of Rho occurred via the inhibition of LMW-PTP, which dephosphorylates p190Rho-GAP and is required for activation of Rho. Consistent with a role for redox regulation of LMW-PTP in cell adhesion and migration, Chiarugi *et al.* showed that levels of ROS increase upon cell adhesion, resulting in inhibition of LMW-PTP and hyperphosphorylation of p125FAK, another substrate of LMW-PTP (19).

Lipid phosphatase PTEN. The lipid phosphatase PTEN shares a number of structural features with the PTPs, including the catalytic site signature motif and the overall three-dimensional structure (42), but it functions as both a lipid phosphatase [dephosphorylating the D3 position of phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3)] (49, 58) and a protein phosphatase (58). Like the other CBPs, PTEN is sensitive to oxidation of its catalytic cysteine. Cell lysates from NIH 3T3 cells that were exposed to H_2O_2 contained a higher concentration of oxidized PTEN in comparison with reduced PTEN, and the oxidized PTEN could be reduced by incubating the lysates with DTT (44). In these studies, thioredoxin reduced PTEN more efficiently than glutaredoxin, and thioredoxin and PTEN coimmunoprecipitated.

Leslie *et al.* adapted the in-gel phosphatase assay method to show that PTEN can be reversibly oxidized *in vivo* (45). They first established that exposing cells to H_2O_2 resulted in reversible inactivation of PTEN, which was detectable in the in-gel phosphatase assay. Using PTEN-null glioblastoma cells, heterologous expression of PTEN was found to reduce PtdIns(3,4,5)P_3, but this activity of PTEN was ablated by H_2O_2 such that levels of PtdIns(3,4,5)P_3 were similar to those in PTEN-null cells. Furthermore, when cells expressing PTEN were exposed to H_2O_2, levels of PtdIns(3,4,5)P_3 increased three- to fourfold. To test for the indirect oxidation of PTEN via the activation of signaling pathways, Leslie *et al.* investigated how combinations of lipopolysaccharide and phorbol 12-myristate 13-acetate would affect PTEN activity in RAW264.7 macrophages. They found that a combination of lipopolysacchaide and phorbol 12-myristate 13-acetate stimulation of cells resulted in an estimated 16% of the cellular PTEN levels becoming oxidized (in comparison with 5% in unstimulated cells). Insulin stimulation of cells, however, did not lead to PTEN inactivation.

Overall concept: mechanistic and molecular consequences of oxidation

Biochemical evidence for reversible oxidation of PTPs. Biochemical studies have also demonstrated that PTPs can be regulated by redox mechanisms. Denu and Tanner showed that H_2O_2 could rapidly inactivate PTP1 (the rat homologue of PTP1B, a cytosolic phosphatase), LAR (a receptor PTP), and vaccinia-1 related phosphatase (VHR; a dual specificity phosphatase) (22). Larger hydroperoxides, which would be excluded from the catalytic cysteine, had no effect on the PTP activity, and inactivation of the PTPs by H_2O_2 could be reversed by thiols such as DTT, β-mercaptoethanol, GSH, or cysteine (22). Significantly, VHR could be exposed to oxidizing agents for up to 3 h and activity could be recovered, suggesting that the oxidative modification of PTPs was stable over prolonged periods. By labeling oxidized and reduced VHR with IA, Denu and Tanner demonstrated that it was the catalytic cysteine of PTPs that became oxidized (22). They based their experiments on work by Zhou *et al.* who had shown previously that the catalytic cysteine of VHR was the only cysteine that could react with IA (96). When H_2O_2-inactivated VHR did not react with IA, they could conclude that oxidation of VHR was occurring at the catalytic cysteine (22).

Catalytic cysteine chemistry. Once it was established that the catalytic cysteines of PTPs could be modified to reversibly oxidized states, it became important to characterize the type of oxidation-induced modification that allows for reversible redox regulation. Under reducing conditions, the catalytic cysteine of PTPs exists as a thiolate anion (Cys-S^-). Cysteine thiolate anions can be readily oxidized to sulfenic acids (Cys-SOH), even under mild oxidative conditions. Cys-SOH groups, however, are highly reactive, and are susceptible to rapid oxidation to the terminally oxidized sulfinic (Cys-SO_2) and sulfonic (Cys-SO_3) acids unless they are stabilized within a protein structure. Sulfenic acids can be protected within proteins by apolar environments or hydrogen bonds (20). Alternatively, cysteines can be "protected" from terminal oxidation by reaction with a thiol to form an inter- or intramolecular disulfide bond. Sulfenic acids and disulfides represent reversibly oxidized states of cysteine. In contrast, there are very few examples in which the formation of Cys-SO_2 and Cys-SO_3 can be reversed. The examples that do exist were only recently discovered, and they involved ATP-dependent enzyme-mediated reduction of a specific protein, peroxiredoxin (9, 91).

Recent biochemical and crystallographic studies indicate that CBPs have evolved a variety of mechanisms to stabilize a reversibly oxidized form of their catalytic cysteine, which suppresses its oxidation to the irreversibly oxidized sulfinic and sulfonic acids, and permits its reactivation. In several

CBPs, including the LMW-PTPs, Cdc25, and PTEN, cysteine residues are located sufficiently close to the catalytic cysteine that oxidation of the catalytic cysteine results in disulfide formation (14, 27, 42, 67). Many other PTPs, however, do not have proximal cysteine residues that would enable intramolecular disulfide bond formation. Intermolecular disulfide bond formation is also unlikely because the catalytic cysteine of PTPs is buried within the catalytic site. This notion is consistent with the results of size-exclusion chromatography and nonreducing SDS-PAGE experiments, which ruled out the formation of intermolecular disulfides in at least PTP1B, VHR, and LAR (22). Based on these and other spectroscopic experiments using 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) to label the catalytic cysteine of VHR, Denu and Tanner proposed that PTPs that lack the ability to form disulfides may form a stabilized sulfenic acid upon oxidation (22).

A cyclic sulfenamide species at the catalytic site of PTP1B facilitates reversible oxidation. To ascertain how the sulfenic acid state was stabilized within a PTP, we determined the crystal structures of catalytic domains of PTP1B (residues 1–321) under various oxidizing conditions (71). In two separate types of experiments, one in which we soaked crystals of PTP1B in H_2O_2 for various time periods, and a second in which we oxidized PTP1B prior to crystallization, we were unable to observe sulfenic acid as a consequence of PTP1B oxidation. Instead, we observed an unexpected intermediate in the oxidation of the catalytic cysteine. In this intermediate, the sulfur atom of the catalytic cysteine (residue 215 in PTP1B) formed a covalent bond to the amide nitrogen of the neighboring serine (residue 216 in PTP1B), a bond that we refer to as the sulfenamide bond (Fig. 5). In the time series of oxidizing PTP1B, we observed mixtures of the thiolate anion state of the catalytic cysteine of PTP1B and the sulfenamide-bonded state when crystals were soaked in H_2O_2 at 4°C for <2 h. Once formed, the sulfenamide bond was stable for >5 h in H_2O_2, indicating that it suppressed cysteine oxidation to sulfinic and sulfonic acids. However, prolonged exposure to oxidants overnight resulted in the conversion of the catalytic Cys to Cys-SO_2 or Cys-SO_3. PTP1B oxidized in solution prior to crystallization also crystallized in the sulfenamide-bonded form. However, over a longer time period, as DTT was not present in these crystallization conditions, the sulfenamide bond oxidized to Cys-SO_3.

Although a sulfenic acid intermediate was not detected in our kinetic crystallographic experiments, generation of the cyclic sulfenamide species, which is dependent on oxidation of Cys215, most likely proceeds via a sulfenic acid intermediate. A likely mechanism would be for the sulfenic acid intermediate to form and for this to promote the nucleophilic attack by the acidic amide nitrogen atom of Ser216 onto the electrophilic Sγ-atom of Cys-OH-215 with concomitant expulsion of water (Fig. 6). The nucleophilicity of the Ser216 amide requires its deprotonation and is probably enhanced by a buried hydrogen bond involving the carbonyl oxygen atom of Cys215 and the side chain of the conserved His214. In independent studies, Jhoti and co-workers also showed that the sulfenamide bond could form in PTP1B crystals, which were incubated with oxidizing inhibitors (87). Significantly, in both studies, formation of the sulfenamide bond in crystals of PTP1B was completely reversible. Soaking crystals overnight in millimolar levels of GSH or DTT was sufficient to reduce the sulfenamide bond. However, when the catalytic cysteine had become oxidized to sulfinic or sulfonic acid, the catalytic cysteine could no longer be reduced by these thiols.

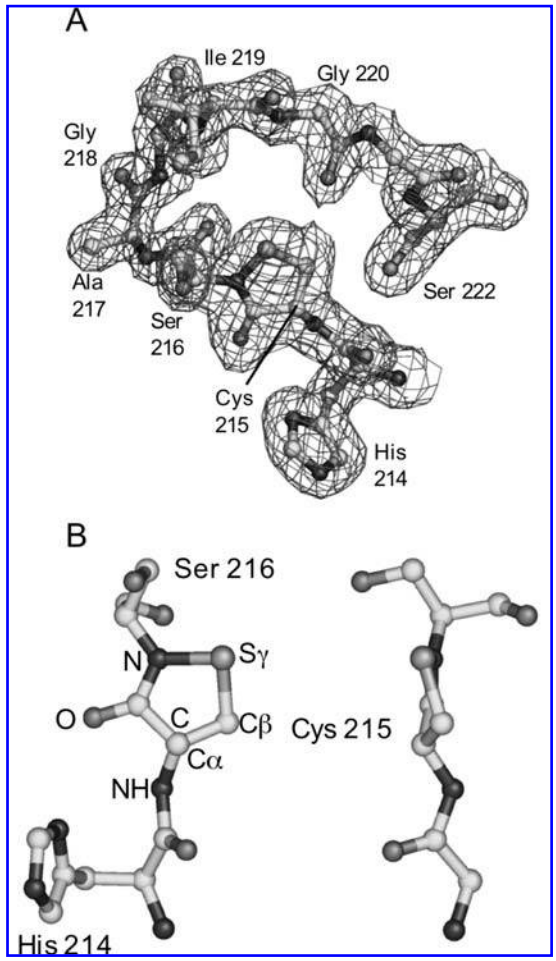

FIG. 5. **(A)** $2F_o$-F_c electron density map for the PTP loop in oxidized PTP1B showing the sulfenamide bond at Cys215 (residues His214 to Ser222 are shown with the side chain of Arg 221 deleted for clarity) (71). **(B)** Two orthogonal views of the cyclic sulfenamide bond.

Studies of cysteine-containing peptides and antibiotic synthesis have provided a precedent for the formation of sulfenamide bonds (isothiazolidinones) via the nucleophilic attack of a nitrogen atom on sulfenic acids (36, 56, 57). In studies on the mechanism by which penicillin sulfoxides are converted to cephalosporin derivatives, Morin and Gordon reported that oxidation of cysteine-containing peptides results in the formation of isothiazolidinones, which are five-membered ring structures containing a sulfenamide bond. They speculated that formation of a sulfenic acid at a cysteine residue within a peptide could lead to the oxidation of the neighboring C-terminal amino acid via formation of an acylimine (*i.e.,* the isomer of a peptide bond in which the amide nitrogen is double-bonded to the carbonyl carbon) and subsequent cycliza-

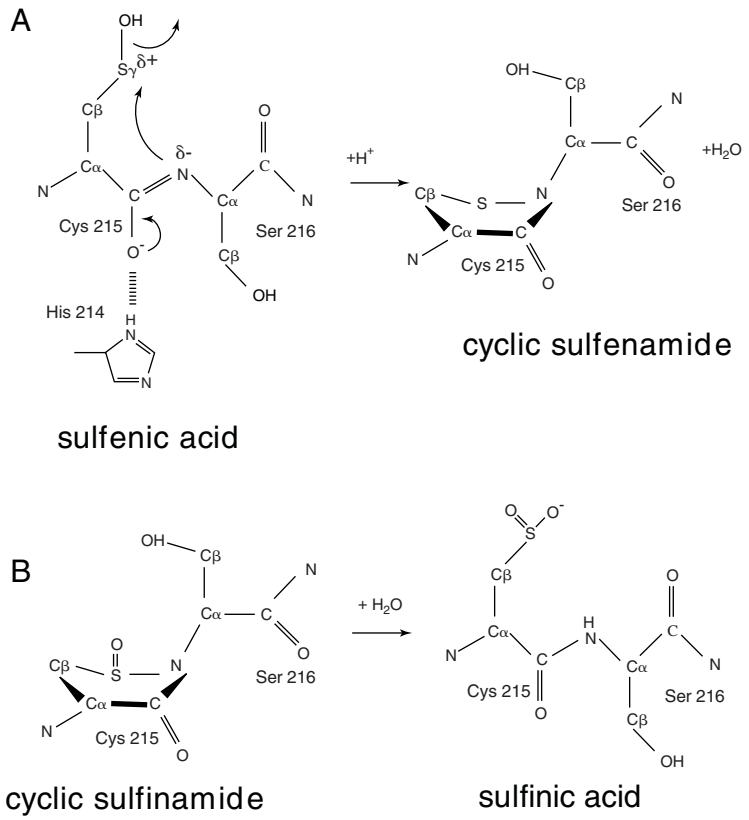

FIG. 6. The proposed mechanism for the formation of the cyclic sulfenamide species and its subsequent oxidation to sulfinic acid. (A) The Sγ-atom of the sulfenic acid intermediate is attacked by the amide nitrogen atom of Ser216, in a condensation reaction, resulting in a sulfenamide bond. **(B)** Oxidation of sulfenamide to sulfinamide and hydrolysis lead to the formation of sulfinic acid.

tion resulting in formation of an isothiazolidinone (56). They also report that isothiazolidones can react with thiols to form disulfides, suggesting that formation of isothiazolidones within proteins could provide a mechanism for disulfide exchange (57). Work by Kim *et al.* on the synthesis of compounds related to the antitumor antibiotic leinamycin also provides a precedent for the intramolecular nucleophilic attack of an amide nitrogen onto a sulfenic acid (36). They propose that amine-catalyzed conversion of 3H-1,2-benzodithiol-3-one 1-oxide to 1,2-benzisothiazolin-3(2H)-one occurs via formation of a sulfenic acid intermediate and subsequent cyclization by nucleophilic attack of an amide toward the sulfenic acid. They provide support for this mechanism by showing that use of a secondary amine, which would block the cyclization reaction, leads to formation of a thiosulfinate, not an isothiazolidinone (36).

The formation of a sulfenamide bond at the catalytic cysteine of PTP1B is accompanied by significant structural rearrangements at the catalytic site of the enzyme (Fig. 7). Upon oxidation, two of the loop regions that are central to catalysis and substrate recognition, the PTP and pTyr loops, change their conformations. Gly218 in the PTP loop shifts by ~7Å, and Tyr46 in the pTyr loop converts from pointing inward toward the catalytic site to becoming completely solvent-exposed, disrupting a hydrogen bond with Ser216. Gln262 in the Q loop also shifts to a different conformation. When the sulfenamide bond becomes oxidized to sulfinic or sulfonic acid, these conformational changes are reversed and the enzyme assumes the same tertiary structure as reduced PTP1B. Thus, the exposure of Tyr46 to solvent and the reorientation of the PTP loop are unique to the sulfenamide bond state of the enzyme. This may provide a mechanism whereby PTP1B containing the sulfenamide bond avoids binding to, and sequestering, its substrates, and that allows it to interact with other proteins in a way that reduced PTP1B or the sulfinic or sulfonic acid states cannot. For example, the adjustment in the conformation of the PTP loop is likely to provide greater accessibility of the catalytic Cys residue to reductants, allowing regeneration of the free thiol. The tyrosine-specific members of the PTP family share highly conserved catalytic domains (mean sequence identity of ~40%) (6), suggesting that formation of the cyclic sulfenamide followed by conformational changes at the catalytic site will be common to other members of the tyrosine-specific PTPs.

Data from studies of PTP1B in solution are also consistent with the formation of the sulfenamide bond and with conformational changes in the enzyme occurring upon oxidation. The mass of the oxidized catalytic domain of PTP1B determined using electrospray mass spectrometry, and the mass of the active-site peptide of PTP1B determined using MALDI mass spectrometry, were both consistent with the expected mass for a sulfenamide-bonded state of the protein (71). In

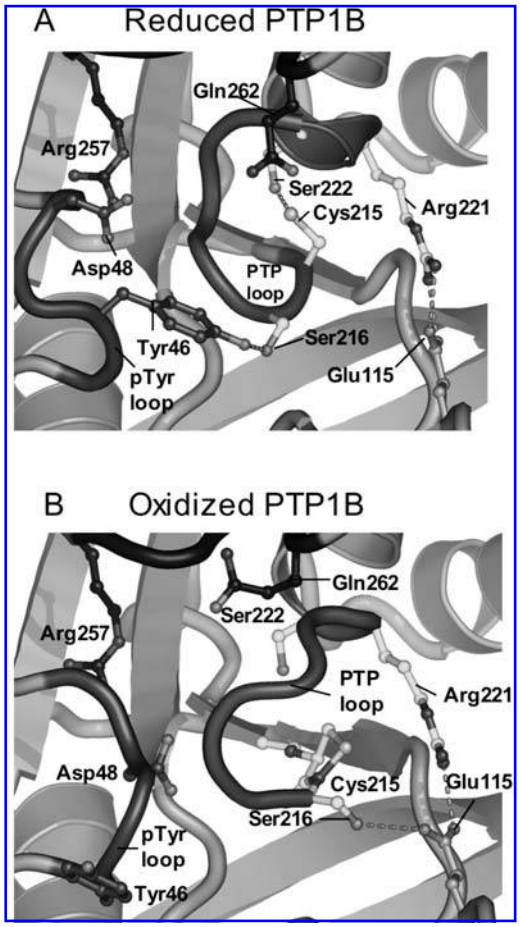

FIG. 7. Oxidation of the catalytic Cys215 of PTP1B results in dramatic conformational changes at the catalytic site (71). Structures of the catalytic site of reduced **(A)** and oxidized **(B)** PTP1B are shown, highlighting the structural changes that occur upon oxidation and formation of the cyclic sulfenamide species. The conformations of the PTP loop and pTyr recognition loop undergo dramatic changes.

addition, a pulse-chase experiment was performed in which PTP1B was first exposed to varying concentrations of $H_2{}^{18}O_2$ that were either sufficient to reversibly oxidize PTP1B or, when used as a control, to irreversibly oxidize PTP1B. The heavy H_2O_2 was then chased with high concentrations of $H_2{}^{16}O_2$, and the mass of the peptide containing the active-site cysteine was analyzed (71). In this experiment, there was no evidence of sulfenic acid at the catalytic cysteine, and the data were consistent with sulfenamide-bond formation in which a water molecule is expelled from the transient sulfenic acid intermediate (Fig. 6).

Studies on the interaction of the catalytic domain of PTP1B with the kinase domain of the IR, a physiological substrate of PTP1B, were also consistent with conformational changes occurring in PTP1B upon oxidation. First, oxidation of a substrate-trapping mutant of PTP1B disrupted its association with the IR kinase. In addition, it has been reported that insulin stimulation of cells can result in the phosphorylation of PTP1B (4, 83). Based on this report and the evidence that PTP1B becomes oxidized after insulin stimulation, we hypothesized that oxidation of PTP1B may also lead to its phosphorylation. As Tyr46 becomes solvent-exposed after oxidation of PTP1B, and Tyr residues are potential substrates for kinases, we tested the hypothesis that this residue was a substrate for the IR kinase. Oxidation of PTP1B promoted its phosphorylation by active IR kinase, but not a mutant of PTP1B where a Phe was substituted for Tyr46. This suggests the hypothesis that oxidation of PTPs *in vivo* may lead to phosphorylation of the PTP, which could affect their interactions with other proteins, such as reducing enzymes or scaffolds. This multitiered regulation of the PTPs is also supported by evidence that wild-type PTP1B associated with the EGF and PDGF receptors in immunoprecipitates from mouse embryo fibroblasts (MEFs) only after the fibroblasts had been pretreated with peroxide (30).

Glutathionylation. In addition to oxidation by H_2O_2, PTP1B and presumably other phosphatases can also become glutathionylated. Both oxidized glutathione (G-S-S-G) and GSH plus diamide can react with PTP1B *in vitro* and result in glutathionylation of the catalytic cysteine (8). Barrett and colleagues propose that irreversible oxidation of PTP1B is prevented *in vivo* through the glutathionylation of the catalytic cysteine. It remains to be seen whether formation of the sulfenamide bond, glutathionylation, or a combination of both will be the relevant mechanism for protecting the catalytic cysteine of PTPs *in vivo*. As the formation of the sulfenamide bond is likely to be a very rapid reaction, it is possible that this could be the first step in protecting the catalytic cysteine, followed by a glutathionylation reaction that can occur as the enzyme is being reduced.

Catalytic site disulfide bonds. Redox regulation of PTEN, LMW-PTPs, and Cdc25C represents an important means of modulating their activities. Interestingly, however, structural and biochemical studies suggest that the consequences of their oxidation differ from that of PTP1B, and a cyclic sulfenamide species, if it forms, is converted to a disulfide bond. These enzymes therefore appear to have evolved a different mechanism to protect their oxidized catalytic sites that involves disulfide bond formation with a vicinal cysteine residue.

PTEN and kinase-associated phosphatase (KAP). Both PTEN and KAP are DSPs. PTEN, as mentioned above, functions as both a lipid phosphatase (49, 58) and a protein phosphatase (58), whereas the substrate of KAP is Thr160 of the CDK2 activation segment (66). DSPs share a marked similarity in structure to PTP1B with identical polypeptide chain topology containing the equivalents of the WPD and Q loops (Fig. 3). They differ from the tyrosine-specific PTPs, however, by having a smaller catalytic domain that lacks the pTyr recognition loop of the PTPs, which is important for conferring the specificity of the tyrosine-specific PTPs for pTyr substrates. On oxidation, the catalytic Cys residue (Cys124) of PTEN forms a disulfide bond with a neighboring Cys residue (Cys79) at the C-terminus of a β-strand (equivalent to β4 of PTP1B) (44). Such a disulfide bond, presumably gener-

ated in response to ROS-mediated conversion of Cys124 to sulfenic acid, would inactivate the phosphatase and protect the cysteine from terminal oxidation to sulfinic and sulfonic acids, and allow its reactivation by reduced thiols. In the structure of PTEN in its reduced state, the Sγ-atom of Cys71 is directed toward the amide group linking Cys124 with Lys125 of the PTP loop, some 6 Å from Sγ of Cys124 (Fig. 3) (42). Rotation of the Cys124 side chain brings these two atoms to a minimum distance of 4.5 Å, still too long for a covalent bond. Formation of a disulfide bond between Cys71 and Cys124 would therefore require conformational changes of PTEN, potentially distorting the PTP loop, with consequent disruption of the phospho-substrate-binding site.

Insights into the structural consequences of PTEN oxidation were revealed from structural studies of KAP (76). Although there is no evidence that KAP is subject to redox regulation *in vivo,* KAP shares with PTEN an equivalent vicinal Cys (Cys79 in KAP). The electron density maps of wild-type KAP revealed clear electron density bridging the side chains of the KAP catalytic cysteine (Cys140) and Cys79, indicating the presence of a disulfide bridge between these two residues (76). Formation of the disulfide bond resulted from spontaneous oxidation of Cys140, probably as a result of the prolonged incubation time required for KAP to crystallize. In oxidized KAP, rotation of the Cys140 side chain to form a disulfide bridge with Cys79 is accompanied by small conformational shifts of the PTP loop. However, the electron density maps are consistent with a sulfate ion of crystallization bound to the catalytic site, suggesting that these small structural perturbations may not disrupt oxyanion binding (Fig. 8A).

Most PTPs do not possess a cysteine residue equivalent to Cys79 of KAP [the exception being PTP-PEST (2)], indicating that similar disulfide bonds would not occur in most PTPs, and favoring the notion that a cyclic sulfenamide species might be the predominant mechanism responsible for stabilizing the oxidized cysteine. Formation of a disulfide in KAP and PTEN does not preclude the possibility that a cyclic sulfenamide species may exist as an intermediate in the generation of a disulfide. In PTP1B, Asn111 is the counterpart of Cys79 of KAP, and comparison of the oxidized PTP1B structure with those of KAP and PTEN shows that formation of the cyclic sulfenamide of PTP1B positions the Sγ-atom of Cys215 in close proximity to the amide side chain of Asn111, indicating that a Cys residue at this position could easily attack the cyclic sulfenamide to form a disulfide via a disulfide-exchange mechanism similar to that proposed by Morin *et al.* (57).

LMW-PTPs. In humans, only one LMW-PTP has been identified, and the origin of this gene family remains uncertain. As discussed below, LMW-PTPs are structurally related to *S. aureus* arsenate reductase, suggesting a potential evolutionary link between LMW-PTPs and enzymes that regulate cellular redox status (55, 74, 77, 93). ROS-dependent oxidation and inactivation of LMW-PTPs is accompanied by generation of a disulfide bond linking the catalytic Cys12 residue with the proximal thiol of Cys17 within the PTP loop (Fig. 3). Generation of this disulfide would be predicted to perturb the conformation of the PTP loop, because formation of the covalent bond cannot be accomplished by simple rotation of the side chains of Cys12 and Cys17, a prediction that has been qualitatively confirmed by fluorescence studies (14). Similarly to the cyclic sulfenamide of PTP1B, a disulfide bond involving the catalytic Cys residue both protects the enzyme from further oxidation to sulfinic acid and facilitates its reduction and reactivation. Ramponi and colleagues demonstrated the critical role of Cys17 to permit reversible oxidation of LMW-PTP *in vivo* (18). In response to PDGF, wild-type LMW-PTP is inactivated by oxidation by 10 min, and cessation of signaling accompanies full restoration of its activity within 40 min. However, a C17A mutant of LMW-PTP, which is as active as the wild-type protein, cannot regain activity following ROS-mediated inactivation, presumably because preventing disulfide formation causes the labile sulfenic acid produced in response to LMW-PTP oxidation to become terminally oxidized to sulfinic acid. These data suggest that intracellular thiols such as GSH and thioredoxin do not react with the catalytic sulfenic acid to prevent its further oxidation. The high susceptibility of Cys12 in the mutant protein to terminal oxidation also suggests that a protective cyclic sulfenamide is unlikely to form in these proteins, despite possessing a similar PTP loop architecture as PTP1B. However, we note that LMW-PTPs do not possess a His residue immediately N-terminal to their catalytic cysteine, which in PTP1B is thought to increase the nucleophilicity of the amide bond that attacks the sulfenic acid intermediate to generate the cyclic sulfenamide (71, 87).

S. aureus arsenate reductase (ArsC), which is markedly reminiscent of LMW-PTPs in architecture despite sharing <20% sequence identity, catalyzes the two-electron reduction of arsenate to arsenite (93) (Fig. 3). Although *S. aureus* ArsC is not known to function as a PTP *in vivo,* it contains a CX_5R motif and is capable of dephosphorylating para-nitrophenyl phosphate, albeit at a very slow rate, some 10^2–10^4-fold less efficiently than conventional PTPs. Reduction of arsenate proceeds via formation of a covalent intermediate between the catalytic Cys10 residue of the PTP loop and arsenate, reminiscent of the cysteinyl-phosphate intermediates of the PTPs (Fig. 4). It is proposed that Cys82 of ArsC attacks the Cys10-arsenate adduct, forming a disulfide. In this process, Cys10 donates an electron pair to the arsenic, reducing it to As(III), and an arsenite ion is liberated. Next, Cys89 attacks Cys82, forming a Cys82-Cys89 disulfide and regenerating the Cys10 thiolate anion. This step is accompanied by major conformational changes of the protein and Cys89 shifts by >10 Å, the result of which oxidative equivalents are brought from the catalytic site to a more solvent-accessible disulfide bond via a disulfide cascade. The Cys82-Cys89 disulfide is subsequently reduced by thioredoxin, regenerating the active ArsC enzyme. Significantly, Cys82 and Cys89 of ArsC are not conserved in the LMW-PTPs, and likewise Cys17 of the LMW-PTPs is not conserved in ArsC, indicating that these two families of enzyme do not share a similar disulfide bond cascade. Interestingly, however, Cys82 of ArsC is structurally equivalent to Cys79 of KAP and Cys71 of PTEN, respectively (Figs. 3 and 8), and therefore the disulfide bond between Cys10 and Cys82 formed as an intermediate in the reaction coordinate of ArsC is equivalent to catalytic site disulfides of oxidized KAP and PTEN (Fig. 8A, C).

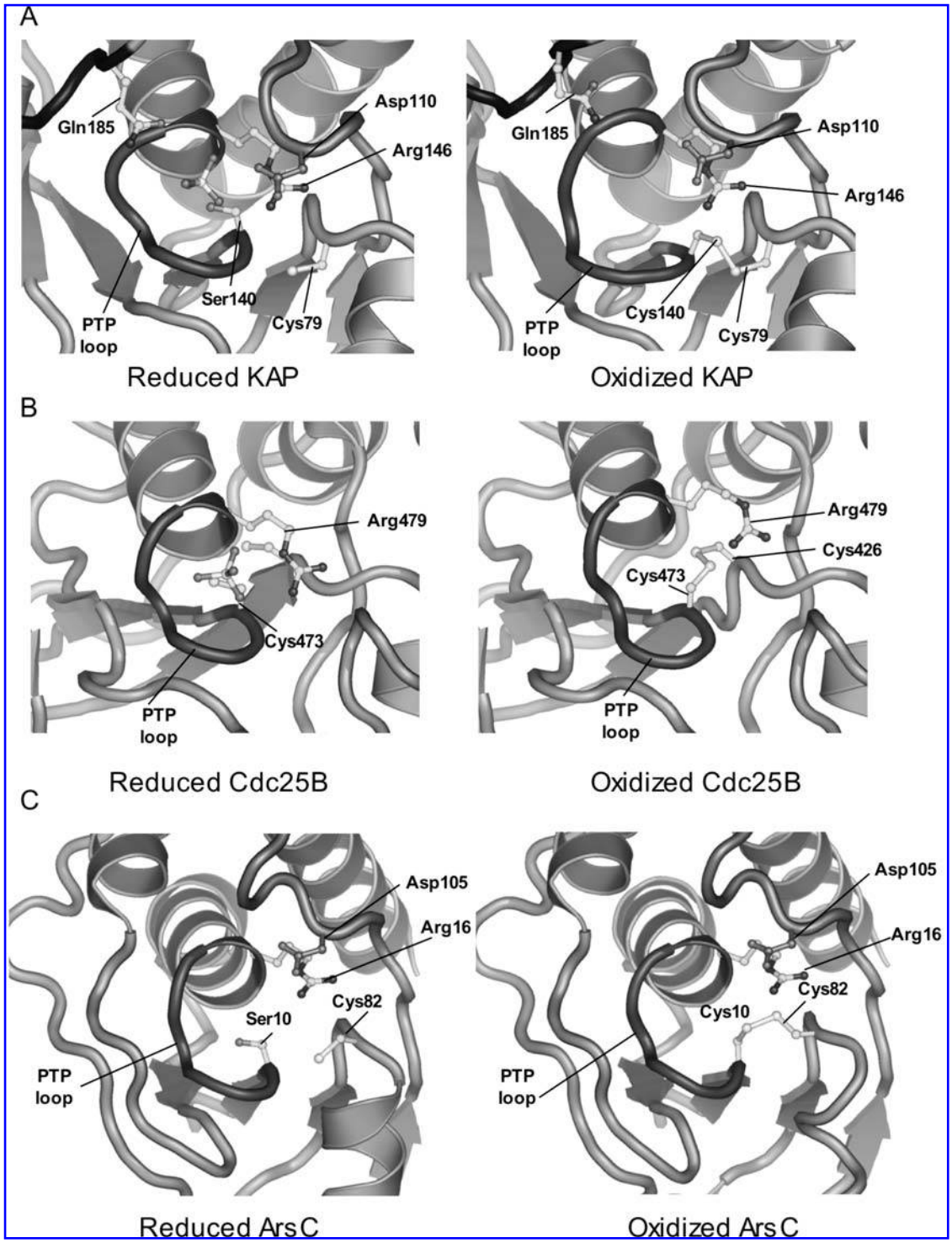

FIG. 8. Oxidation of the catalytic Cys residues of (A) KAP (76), (B) Cdc25B (67), and (C) ArsC (55) results in formation of a disulfide bond. A distortion of the conformation of the PTP loop of Cdc25B results in the dissociation of the sulfate anion from the catalytic site.

Cdc25. The physiological role of reversible redox regulation of the Cdc25 family is not as unequivocal as for members of the PTP and LMW-PTP families. However, inactivation of Cdc25 via catalytic site oxidation may provide a means for oxidative stress signals to delay cell-cycle progression. Recent studies have demonstrated that oxidation of Cdc25C promotes its degradation *in vivo* and increases interactions with 14–3-3 proteins (73). Biochemical studies on Cdc25B and Cdc25C have revealed that the catalytic Cys residue of these proteins is remarkably sensitive to oxidation by H_2O_2

(75). In some crystal structures of the catalytic domains of Cdc25A and Cdc25B, the catalytic Cys residue was oxidized to a disulfide bond with a proximal Cys residue (Cys426 of Cdc25B) (27, 67). Other oxidized species of cysteine, for example, sulfenic and sulfinic acids, were not observed (Figs. 3 and 8). Cys426 is located on a β-strand, adjacent to the PTP loop, in a similar position to Cys79 of KAP. In Cdc25B, formation of the disulfide bond caused subtle conformational perturbations of the PTP loop, with concomitant expulsion of the oxyanion ligand from the catalytic site (Fig. 8B) (67). Thus, similar to PTP1B, oxidation of Cdc25 both inactivates the enzyme and disrupts substrate recognition.

In a detailed kinetic analysis of Cdc25B oxidation, Sohn and Rudolph (75) demonstrated that, on oxidation of the catalytic Cys residue (Cys473), the sulfenic acid product is rapidly converted into a disulfide with Cys426. This intramolecular reaction is substantially faster than secondary oxidation of Cys-OH-473, so that formation of sulfinic acid is suppressed. Cys426 therefore appears to play a similar role to equivalent Cys residues in PTEN and KAP, Cys17 of LMW-PTPs, and the cyclic sulfenamide of PTP1B, that is, to protect the oxidized cysteine from terminal oxidation to sulfinic acid. Consistent with this hypothesis, in a mutant of Cdc25B with an Ala substitution of Cys426, Cys473 was readily oxidized to sulfinic acid (75). Moreover, although in wild-type Cdc25B, the sulfenic acid intermediate was too transient to be observed before its conversion to a disulfide, in the C426A mutant, the inability to form a disulfide allowed transient observation of the sulfenic acid prior to its oxidation to sulfinic acid. This finding is reminiscent of the transient properties of the peroxide-generated sulfenic acid at the catalytic site of PTP1B, prior to its conversion to the cyclic sulfenamide species (71). In the PTP1B kinetic crystallographic oxidation experiment, sulfenic acid was not detected en route to the sulfenamide species, suggesting that its formation was rate-limiting. However, this experiment lacks the sensitivity to detect a small fraction of PTP1B with sulfenic acid at the catalytic site. Evidence for the presence of sulfenic acid in PTP1B in response to H_2O_2 oxidation was obtained in previous studies using the labeling reagent NBD-Cl, which reacts with sulfenic acid to generate a sulfoxide derivative (7). After exposure of PTP1B to H_2O_2 and NBD-Cl followed by trypsin digestion, a peptide was isolated, which contained the catalytic site Cys residue covalently modified by a sulfoxide derivative of NBD-Cl, consistent with sulfenic acid formation.

CONCLUDING REMARKS

ROS are now recognized as important mediators of cellular signaling processes. Current research is focused on identifying effectors of ROS, understanding mechanisms of signaling specificity and reversibility, and defining the consequences of ROS on signaling pathways. The CBPs have emerged as important targets of ROS signaling. The diverse roles that these enzymes play in regulating virtually all cellular functions by determining the balance of intracellular protein phosphorylation suggests ubiquitous roles for redox regulation as a signaling mechanism. A combination of biochemical and crystallographic studies has revealed how different CBPs have evolved related, but nevertheless distinct, structural solutions to permit reversible redox regulation of protein activity and to protect the oxidized catalytic cysteine residues from terminal oxidation. The dramatic structural rearrangements that occur at the catalytic site of PTP1B concomitant with formation of the cyclic sulfenamide species imply that redox regulation may also allow for multitiered regulatory mechanisms, such as oxidation-induced conformational changes exposing sites of phosphorylation. Redox-dependent protein conformational changes *per se* also have the potential to regulate a variety of cell processes. For example, it seems likely that the catalytically inactive D2 domains of the RPTPs function as redox sensors, responding to ROS generation by undergoing protein conformational changes capable of initiating further signaling events. Thus, signal transduction pathways have evolved a number of ways to exploit the sensitivities of CBPs to oxidation, and more are likely to be discovered as the intricacies of these redox-regulatory mechanisms continue to be unraveled.

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ABBREVIATIONS

AA, arachidonic acid; ArsC, arsenate reductase; CBP, cysteine-based phosphatase; Cys-SOH, cysteine-sulfenic acid; Cys-SO_2, cysteine-sulfinic acid; Cys-SO_3, cysteine-sulfonic acid; DSP, dual-specificity phosphatase; DTT, dithiothreitol; EGF, epidermal growth factor; G-CSF, granulocyte colony stimulating factor; GSH, glutathione; H_2O_2, hydrogen peroxide; IA, iodoacetamide; IR, insulin receptor; KAP, kinase-associated phosphatase; LAR, leukocyte common antigen-related phosphatase; LMW-PTP, low-molecular-weight protein tyrosine phosphatase; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; NO, nitric oxide; ONOOH, peroxynitrite; PDGF, platelet-derived growth factor; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PTP, protein tyrosine phosphatase; PTP1B, protein tyrosine phosphatase 1B; pTyr, phosphotyrosine; ROS, reactive oxygen species; RPTP, receptor protein tyrosine phosphatase; RSNO, *S*-nitrosothiols; RTK, receptor tyrosine kinase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; VHR, VH-1 related phosphatase.

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