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Review

PTPs versus PTKs: The redox side of the coin

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Abstract
The phosphorylation of tyrosine, and to a lesser extent threonine and serine, plays a key role in the regulation of signal transduction during a plethora of eukaryotic cell functions, including cell activation, cell-cycle progression, cytoskeletal rearrangement and cell movement, differentiation, apoptosis and metabolic homeostasis. *In vivo*, tyrosine phosphorylation is reversible and dynamic; the phosphorylation states are governed by the opposing activities of protein tyrosine kinases (PTKs)[2] and protein tyrosine phosphatases (PTPs). Reactive oxygen species (ROS) act as cellular messengers in cellular processes such as mitogenic signal transduction, gene expression, regulation of cell proliferation, senescence and apoptosis. Redox regulated proteins include PTPs and PTKs, although with opposite regulation of enzymatic activity. Transient oxidation of thiols in PTPs leads to their inactivation by the formation of either an intramolecular S–S bridge or a sulfenyl–amide bond. Conversely, oxidation of PTKs leads to their activation, either by direct SH modification or, indirectly, by concomitant inhibition of PTPs that guides to sustained activation of PTKs. This review focuses on the redox regulation of both PTPs and PTKs and the interplay of their specular regulation.

Keywords: *protein tyrosine phosphatases, protein tyrosine kinases, reactive oxygen species, redox, anchorage-dependent cell growth*

Abbreviations: *ECM, extracellular matrix; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; ERK, Extracellular signal regulated kinase; FAK, focal adhesion kinase; GF, growth factor; LMW-PTP, Low molecular weight-phosphotyrosine phosphatase; LOX, 5-Lipoxygenase; IRK, Insulin receptor kinase; MEN, multiple endocrine neoplasia; NO, nitric oxide; PDGF, platelet-derived growth factor; PDGF-R, platelet-derived growth factor receptor; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; PTEN, Phosphatase and tensin homologue deleted on chromosome ten; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; R-PTPα, Receptor protein tyrosine phosphatase α; RTK, receptor tyrosine kinase; SHP2, Src homology phosphatase 2; TC45, T cell PTP, fragment of 45kDa; UV, ultraviolet*

PTP family:

Protein tyrosine phosphatases (PTPs) can exert both positive and negative effects on a signaling pathway and play crucial physiological roles in a variety of mammalian tissues and cells[1]. It is important to note that, as observed with protein tyrosine kinases (PTKs), deregulation of PTP activity can also contribute to the pathogenesis of many human diseases[2,3].

PTPs are a family of enzymes, whose structural diversity and complexity rival those of PTKs. Unlike PTKs, however, which share sequence homology with protein serine/threonine kinases, PTPs show no sequence similarity with the protein serine/threonine phosphatases. The PTP superfamily shares a common CX_5R active site motif and an identical catalytic mechanism. In addition to the catalytic domain, PTPs are highlighted with a wide range of structural elements including SH2 domains, PDZ domains, extracellular ligand binding domains and many others[2,4]. A recent estimation from the nearly completed human genome sequence suggested that humans have 112 PTPs[5], which include both the tyrosine-specific and dual-specific phosphatases. The tyrosine-specific phosphatases, such as PTP1B,

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hydrolyze phosphotyrosine-containing proteins, while the dual-specific phosphatases, such as Cdc25, can utilize protein substrates that contain phosphotyrosine, as well as phosphoserine and phosphothreonine[6]. The tyrosine-specific PTPs can be further divided into two clusters: receptor-like (RPTPs) and cytosolic PTPs (cPTPs). RPTPs, exemplified by RPTPα and CD45, generally have an extracellular ligand-binding domain, a single transmembrane region, and one or two cytoplasmic PTP domains. The intracellular PTPs, exemplified by PTP1B, LMW-PTP and SHP2, contain a single catalytic domain and various amino or carboxyl terminal extensions and may contain SH2 domains that have targeting or regulatory functions. Examples of dual-specificity phosphatases include the mitogen activated protein kinase (MAPK) phosphatases, the cell cycle regulators Cdc25 phosphatases, and the tumor suppressor PTEN[4,5]. All PTPs are characterized by their sensitivity to vanadate, ability to hydrolyze *p*-nitrophenyl phosphate, insensitivity to okadaic acid, and lack of metal ion requirement for catalysis[7].

The PTP active site is located within a fissure (~9 Å profound for the tyrosine-specific PTPs and ~6 Å profound for the dual-specificity phosphatases) on the protein outside. The much deeper active site pocket in the tyrosine-specific phosphatases selects solely pTyr-containing substrates[8], while the more superficial active site crevice of the dual-specificity phosphatases may contain both pTyr and pSer/pThr[9,10]. The PTPs use covalent catalysis (Figure 1A), utilizing the thiol group of the active site cysteine as the attacking nucleophile, to form a cysteinyl-phosphate enzyme intermediate (E-P)[11,12]. Mutagenesis-directed substitutions of the Cys residues completely abrogate PTP activity. The nucleophilic cysteine is accommodated within the active site, specifically designed to bind a negatively-charged substrate. Both the E-P formation and hydrolysis is supported by a conserved aspartic acid[13]. Thereafter PTPs speed up the formation and hydrolysis of E-P by preferentially binding the transition states with the positively charged chain of the active site arginine residue[11,13].

RPTPs are mainly regulated through dimerization. Dimerization is an important regulatory mechanism for many signal transduction molecules, in particular transmembrane receptor proteins[14]. Extensive studies have established that ligand-induced dimerization (or oligomerization) is critical for the activation of receptor tyrosine kinases, antigen receptors and cytokine receptors. In most cases, dimerization activates receptor kinase activity, either intrinsic or associated, by trans- or autophosphorylation[15]. Conversely, RPTPs are often inhibited by dimerization, but in most cases, the ligands that induces such a regulation have not been identified[14]. The current model for negative regulation of RPTPs has been elucidated for both CD45 and RPTPα. In the monomeric state, CD45 or RPTPα are active and "prime" a Src Family Kinase (Lck or Src) by dephosphorylating their inhibitory tyrosine (see cytoplasmic PTK regulation). Dimerization of the RPTP causes its inactivation through the symmetrical interactions between the catalytic site of phosphatase domain and the inhibitory structural wedge[16].

Cytoplasmic PTPs display a more complex regulation. SH2 domain-containing PTPs, exemplified by SHP-1 and SHP-2, contain one or two SH2 domains at the N-terminus, a PTP domain and a C-terminal tail[17]. As for several SH2-domain containing enzymes, that are catalytically inactive as a consequence of SH2 domain mediated auto inhibition, the PTPs are essentially inactive under basal conditions. In fact, during resting circumstances the N-terminal SH2 domain of SHP-2 is inserted into the catalytic cleft, thus resulting in auto inhibition of the PTP domain. The displacement of auto inhibiting SH2 domain of SHP-2 by tyrosine phosphorylated ligands dramatically increases PTP activity, due to aperture of the enzyme conformation[18]. To note, both SHPs undergo phosphorylation at both C-terminal tyrosyl residues in response to some growth factors[19]. Tyrosine phosphorylation is common to all cytosolic PTPs, including non-SH2-containing PTPs, exemplified by PTP1B and LMW-PTP. Both these phosphatases have been reported to be activated by phosphorylation at Tyr66 for PTP1B and Tyr131 for LMW-PTP, respectively[20,21].

Although RPTPs and cytoplasmic PTPs display different mechanisms of regulation, both dimerization and tyrosyl-phosphorylation being highly specific for each subclass, they share a further common regulatory mechanism: the reversible oxidation of catalytic cysteine, leading to inhibition of catalytic activity.

PTK family:

PTKs are commonly divided into two families, the transmembrane receptor family and the non-receptor family, which are estimated to comprise 58 and 32 members in the human genome, respectively[22]. Receptor and non-receptor PTKs are essential enzymes in cellular signalling processes that regulate cell growth, differentiation, migration and metabolism. The kinase activity of PTKs is tightly controlled through steric, autoregulatory mechanisms, as well as by the action of protein tyrosine phosphatases. Recent structural studies have revealed several modes of autoregulation governing the catalytic state of these enzymes. Aberrant catalytic activity of many PTKs, via mutation or overexpression, plays an important role in numerous pathological conditions, including cancer[22]. Receptor PTKs (RPTKs) are cell-surface, transmembrane receptors possessing a multidomain extracellular portion that binds polypeptide ligands, a single-pass transmembrane helix, and

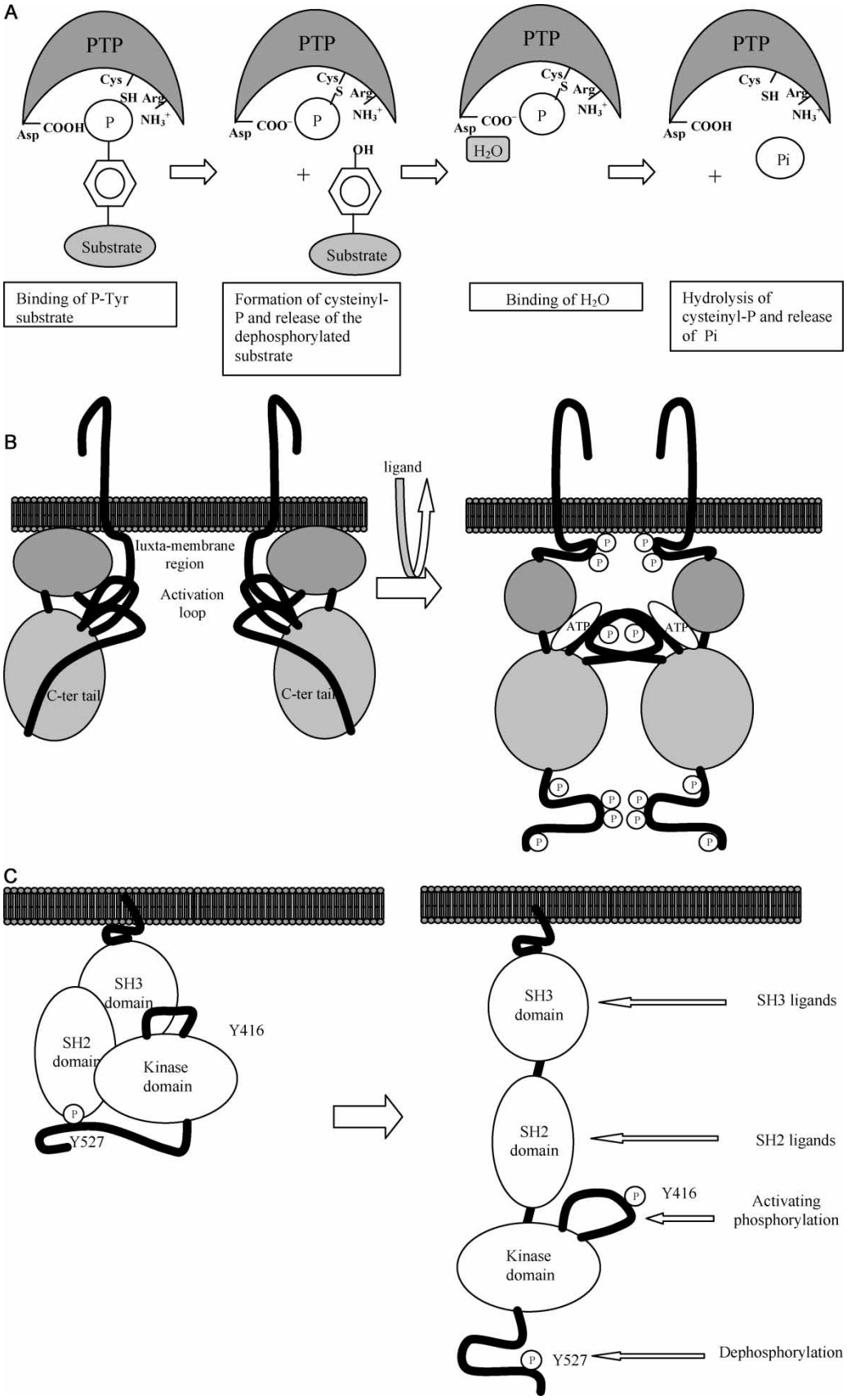

Figure 1. PTP and PTK activity. A: PTP catalytic mechanisms: the PTP reaction is nucleophilic in nature and is composed of at least two chemical steps: the formation and breakdown of the phosphoenzyme intermediate (E–P). The active-site Arg plays a key role in substrate binding, through the interaction of its positively charged guanidino group with substrate's phosphate moiety. The active site nucleophile corresponds to the invariant Cys residue in the PTP signature motif. The invariant Asp residue acts first as a general acid

a cytoplasmic portion containing a tyrosine kinase domain and regulatory sequences both N- and C-terminal to the kinase domain[23]. About one-third of tyrosine kinases are classified as non-receptor tyrosine kinases. They are found in the cytoplasm, lack a transmembrane section, and generally function downstream of the receptor tyrosine kinases. The RTK family includes, among others, epidermal growth factor (EGF) receptor, platelet-derived growth factor (PDGF) receptor, fibroblast growth factor receptor and the insulin receptor. Examples of the non-receptor, i.e. cytoplasmic PTK (cPTK) family include Src, Abl, Focal adhesion kinase (FAK) and the Janus kinases. For appropriate subcellular localization, cPTKs typically contain modular domains that mediate protein–protein (e.g. SH2/SH3 domains) or protein–lipid interactions (e.g. pleckstrin homology [PH] domain)[24], or are lipid-modified (e.g. myristoylated)[25]. In general, tyrosine autophosphorylation serves to stimulate the catalytic activity of PTKs and to generate docking sites for recruitment of substrate proteins[26].

The tyrosine kinase domain of PTKs spans approximately 300 residues and adopts a two-domain architecture that is shared by the protein serine/threonine kinase family. It consists of an N-terminal lobe comprising a five-stranded β-sheet, one α-helix and a larger C-terminal lobe. ATP binds in the cleft between the two lobes and the tyrosine-containing segment of a protein substrate interacts with residues in the C-terminal lobe. Several non-contiguous polypeptide segments of the kinase domain contribute to the formation of the active site, including the nucleotide-binding loop, and the catalytic and activation loops in the C-terminal lobe[23].

Most PTKs are maintained in a low (basal) activity state through various autoregulatory mechanisms, all of which prevent the optimal configuration of the aforementioned polypeptide segments at the kinase active site. Although the conformation of the catalytic loop is remarkably similar from one PTK to the next, and between inactive and active states, other segments, in particular α-helix C and the activation loop, are often switch elements in intrasteric regulation. In general, activation of receptor PTKs is achieved through ligand binding to the extracellular domain, which stabilizes a dimeric receptor arrangement, facilitating *trans*-phosphorylation in the cytoplasmic domain[27]. Evidence indicates that RPTK dimerization *per se* is not always enough for kinase activation. An additional requirement for ligand-induced conformational switches has been indicated, ensuring that the catalytic domains are put beside in a suitable configuration to allow phosphorylation in *trans* involving receptor subunits[1,27]. For some RPTKs, including PDGF receptor, Kit/stem-cell factor receptor, colony-stimulating factor-1 receptor, ephrin receptors and insulin receptor, the juxtamembrane segment has been implicated in auto inhibition. Therefore, autophosphorylation of one or two homologous juxtamembrane tyrosines in these receptors is necessary for complete kinase activation, and mutation to phenylalanine appreciably reduces ligand-evoked kinase activation (Figure 1B)[28].

For cPTKs, the activation mechanisms are more complex than for RPTKs, involving heterologous protein–protein interactions, as well as clustering to enable *trans*-phosphorylation (Figure 1C). The Src family kinases are the prototypical non-receptor cPTKs, and their structure has been studied extensively (for a review see[29]). The concerted intramolecular interactions between the SH2 domain and the phosphorylated C-terminal tail, and between the SH3 domain and the SH2-kinase linker, avoid the kinase domain from adopting an active configuration[30]. The SH2 domain interacts with phospho-Tyr 527 in the C terminus and the SH3 domain with the polyproline helix in the region near the SH2 domain. This causes misalignment of residues that are critical for kinase activity. The binding of ligands to the SH2 or SH3 domain and/or dephosphorylation of phospho-Tyr527 by PTPs relieves the inhibition on the kinase, leading to autophosphorylation of Tyr416, located in the activation loop. Several PTPs have been indicated to dephosphorylate the inhibitory Tyr527, including RPTPα, PTPλ and RPTPε, PTP1B, SHP-1 and SHP-2. The enzyme autophosphorylated in the stimulatory tyrosine is a fully active enzyme[31].

Beside SH2/SH3 and autophosphorylation global switches, several redox-linked signal transduction pathways have been recently proposed as concurrent mechanisms for protein tyrosine kinase activation.

during E–P formation, probably by protonating the leaving group, and must be protonated for optimal PTP activity. Thereafter, the same invariant Asp residue acts as a general base to activate the nucleophilic water in the E–P hydrolysis step. B: RTK activation. Left: RPTK kinase activity is firmly repressed under unstimulated conditions, where the activation loop and the juxtamembrane and C-terminal loops completely preclude ATP access to the catalytic site. Right: ligand-induced receptor dimerization and tyrosine autophosphorylation result in relief of the inhibitory constraints exerted by the activation loop, and the juxtamembrane and C-terminal regions. In addition, the juxtamembrane and C-terminal region might further impede substrate access to catalytic cleft. C: c-Src activation. Left: c-Src kinase activity is tightly inhibited in the unstimulated state through the SH2 domain binding of P-Tyr 527. This causes misalignment of residues that are essential for kinase activity. Right: binding of ligands to the SH2 or SH3 domain and/or dephosphorylation of P-Tyr527 by PTPs relieves the inhibitory constraints on the kinase, finally resulting in autophosphorylation of Tyr416 in the activation loop.

Reactive oxygen species and their sources:

During the past decade, reactive oxygen species (ROS), have been identified as significant mediators of cell growth, adhesion, differentiation and apoptosis. Proteins with low-pK_a cysteine residues, which are susceptible to oxidation by ROS embrace several transcription factors as the nuclear factor κ-B[32], activator protein 1[33], hypoxia-inducible factor[34], p53[35], the p21Ras family of proto-oncogenes[36] and, of note, many PTPs and PTKs.

ROS cover a range of partially reduced metabolites of oxygen (e.g. superoxide anions, hydrogen peroxide and hydroxyl radicals) possessing higher reactivity than molecular oxygen. ROS can be derived from exogenous sources or produced as a consequence of the exposure to some environmental challenges. Inside cells, ROS are generated through a variety of processes, for example, as by-products of normal aerobic metabolism, or as second messengers in various signal transduction pathways, including integrin engagement[37]. Superoxide anions are a by-product of mitochondrial electron chain flux, accounting for ~2% of total oxygen utilization by the organelle[38]. The physiological activity of the respiratory chain leads to the production of semi-quinones, a potential source of ROS[39]. The respiratory chain produces ROS at complex I (NADH/ubiquinone oxidoreductase) and complex III (ubiquinol/cytochrome *c* oxido-reductase). The ubiquinone site in complex III appears as the major site of mitochondrial ROS production: this site catalyzes the conversion of molecular oxygen to the superoxide anion radical by a single electron transfer to molecular oxygen.

Superoxide anions may also be synthesized enzymatically by NADPH oxidase activity, prostanoid metabolism by cycloxygenases and lipoxygenases, xanthine oxidase, catecholamine autooxidation and the NO synthases. Although it remained unclear for many years, whether non-phagocytic cells contain an NADPH oxidase system comparable to phagocytic one, numerous types of NADPH oxidases have been identified in fibroblasts and other non-phagocytic cells[40,41]. NADPH oxidase is a protein complex formed by membrane (gp91phox, p22phox) and cytosolic (Rac, p67phox, p47phox, p40phox) proteins[42]. Membrane oxidases similar to the phagocytic NADPH oxidase complex are expressed almost ubiquitously in non-phagocytic cell types[43]. NADPH oxidase catalyses the one-electron-reduction of O_2 to $O_2^{\cdot-}$, which spontaneously or enzymatically catalyzed dismutes to H_2O_2. Several lines of evidence reveal that NADPH oxidase is specifically involved in the generation of ROS by soluble GFs, cytokines or other signalling molecules such as insulin[48], PDGF and EGF[49,50], transforming growth factor-β1[44,45], or by interleukin-1[46], tumor necrosis factor-α[47], angiotensin II[51], thrombin and lysophosphatidic acid[52].

5-Lipoxygenase (LOX) is a mixed function oxidase involved in the synthesis of leukotrienes from arachidonic acid, and its activation is usually followed by translocation to the nuclear envelope. Arachidonic acid induces membrane ruffling and H_2O_2 production in a Rac1-dependent, manner. Indeed, arachidonic acid is generated via Rac-mediated phospholipase A2 activation in response to GFs and cytokines and is implicated in cell growth and gene expression. Arachidonic acid can be metabolized to leukotrienes and prostaglandins by LOX and cyclooxygenase, respectively. These metabolites are involved in the regulation of EGF-induced actin remodelling and are necessary and sufficient for the formation of actin stress fibre[53]. Leukotrienes were also known to play an important role in the signalling pathway regulating cellular survival and apoptosis[53].

Transient fluctuations in ROS provide important regulatory functions, but when present at high and/or sustained levels, they can cause severe damage to DNA, protein and lipids. A number of defense systems have evolved to contrast the accumulation of ROS. These include various non-enzymatic molecules (e.g. glutathione, vitamins A, C and E and flavonoids) as well as enzymatic defense systems (e.g. superoxide dismutases, catalase and glutathione peroxidases). Regrettably, these defence mechanisms are not always sufficient to counteract the production of ROS, resulting in what is termed a state of oxidative stress. Oxidative stress has been implicated in a wide variety of disease processes including cancer, atherosclerosis, diabetes, pulmonary fibrosis, neurodegenerative disorders and arthritis, and is believed to be a key factor in aging[54].

Redox regulation of PTPs:

Oxidation of PTP active-site cysteine to cysteine sulfenic derivative by various oxidant agents, including H_2O_2, leads to their enzymatic inactivation, although this modification can be reversed by incubation with thiol compounds[55–57]. These observations suggest that oxidation of catalytic cysteine of PTPs may take place *in vivo* in response to ROS or to an increase in redox potential. Numerous papers have provided insight into how PTPs might transduce oxidative stress conditions, leading to a perfect redox regulation of these enzymatic class. Rhee and colleagues demonstrated that EGF stimulation of A431 cells induces a burst of ROS whose time course inversely correlates with the activity of PTP1B[57]. Subsequent studies demonstrated that the cysteine-sulfenic intermediate formed by oxidation of PTP1B could in the presence of glutathione form a mixed disulfide[58]. The formation of mixed disulfides, also known as glutathionylated proteins, has recently been

recognized as an additional protein modification by physiological oxidative stress[59]. A reversible oxidation was then demonstrated for Low Mw-PTP (LMW-PTP) throughout PDGF stimulation[60,61]. Both PTP1B and LMW-PTP completely rescue their phosphatase activity due to re-reduction after 30 min from receptor activation. Thereafter, the reversible oxidation of SHP-2 following PDGF administration has also been described by Meng et al. using a functional modified in gel-PTP assay[62]. Beside, Rhee and colleagues point up intramolecular S–S bridge-dependent, *in vitro* redox regulation of the lipid phosphatase PTEN[63], while Savintsky provide evidence for Cdc25C phosphatase degradation triggered by H_2O_2-induced disulfide bond formation[64]. In addition, H_2O_2-dependent, oxidation stabilizes dimers of transmembrane PTP-α (RPTPα), resulting again in phosphatase inactivation[65], by inducing intermolecular disulfide bond formation. Finally, a study of insulin receptor tyrosine phosphorylation by PTPs by means of modified in gel PTP assay, demonstrated that stimulation of cells with insulin resulted in the rapid and transient oxidation and inhibition of PTP1B and TC45, the 45-kDa splice variant of the T cell protein-tyrosine phosphatase[66]. Beside PTP redox regulation linked to GF signalling, a specific role of oxidants in integrin receptors signalling has now emerged. ECM-induced ROS actually behave as integrin second messengers as they dramatically affect cytoskeleton assembly by transiently oxidizing redox sensitive proteins. In response to ECM contact, and in strict concomitance with the ROS burst, LMW-PTP is oxidized/inhibited, thereby preventing the dephosphorylation of two key regulators of cytoskeleton dynamics: the focal adhesion kinase (FAK)[37] and a GTPase activating protein for the GTPase Rho (p190RhoGAP)[67]. Hence, upon integrin engagement, the redox circuitry leads, as a final event, to a properly executed cell adhesion and spreading onto fibronectin.

A general trait of PTP redox regulation is the formation of an S–S bridge containing the catalytic site cysteine, which is thus preserved from extra oxidation (Figure 2A). As outlined above, different cysteine residues can take part in the formation of disulfide bonds during PTP oxidation. For example, cysteine disulfide may derive from two cysteines within the catalytic site as for LMW-PTP [60,61], between distant cysteines as for Cdc25[64], RPTPα[65] and PTEN[68], as well as between PTPs and glutathione in the case of PTP1B[58]. Very recently an additional mechanism to prevent hyperoxidation of catalytic cysteine in PTPs has been reported: the reactive cysteine of PTP1B is oxidized to Cys-SOH and then converted into sulfenic-amide intermediate[69,70]. Thus, the common mechanism to all redox regulated PTPs to prevent further and irreversible oxidation, is to block Cys-SOH by S–S disulfide (either with intramolecular cysteine or by GSH mixed disulfide formation) or by a transient S–N sulfenyl–amide bond (Figure 2A)[71,72].

While not yet identified, intracellular reducing agents such as thioredoxin, glutaredoxin and reduced glutathione are thought to play essential roles in the recovery of oxidized PTP activity (Figure 2A)[72]. Considering the differences in the p*K*a values of catalytic cysteines among PTPs[73], we propose that the availability as thiolate ions and the structural distance between the two cysteines involved in the disulfide may powerfully influence the ability of a specified phosphatase to be rapidly regulated by changing intracellular redox conditions. This may represent a degree of specificity for PTP redox regulation and lead to different oxidation and/or rescue among different PTPs and among different pathophysiologic circumstances.

Redox regulation of PTKs:

Oxidants can also modulate the activity of protein kinases and, among these, PTKs. Again in contraposition to PTPs, but in agreement with their opposing role in the modulation of protein tyrosine phosphorylation, PTKs are activated by oxidation. However, the activation for some kinases appears to be essentially due to two mechanisms. First, similar to what happens for PTPs, cysteine oxidation may occur, leading to direct kinase activity enhancement (Figure 2B). Second, as PTKs themselves are frequently tyrosine phosphorylated proteins and their activity is increased due to phosphorylation, most likely the concomitant inhibition of PTPs indirectly leads to sustained activation of PTKs.

Oxidation-dependent activation of PTKs may engage membrane bound kinases, as RTKs, or intracellular kinases, as Src tyrosine kinases, FAK, jointly with many other Ser-Thr or dual specificity kinases, such as ERK or Akt and Ask[74]. A direct oxidation of RTKs has been reported for insulin receptor kinase (IRK), EGF-R, PDGF-R and Ret kinase. Schmid et al. have reported that best insulin sensitivity may need a process of 'redox priming' of the beta subunit of IRK, likely due to a decrease in IR beta-chain sulfhydryl groups due to oxidation[75]. In addition, it has been reported that 'redox priming' of the IRK facilitates its autophosphorylation in the activation loop. In fact, 3-D models of IRK revealed that the oxidation of any of the four cysteine residues 1056, 1138, 1234 and 1245 into sulfenic acid produces structural changes that bring Tyr1158 into close proximity with Asp1083 and make the catalytic site at Asp1132 and Tyr1162 more accessible[76]. Hence, it is likely that cysteine oxidation enhances the tyrosine kinase activity of the IRK, thereby potentiating its downstream signalling.

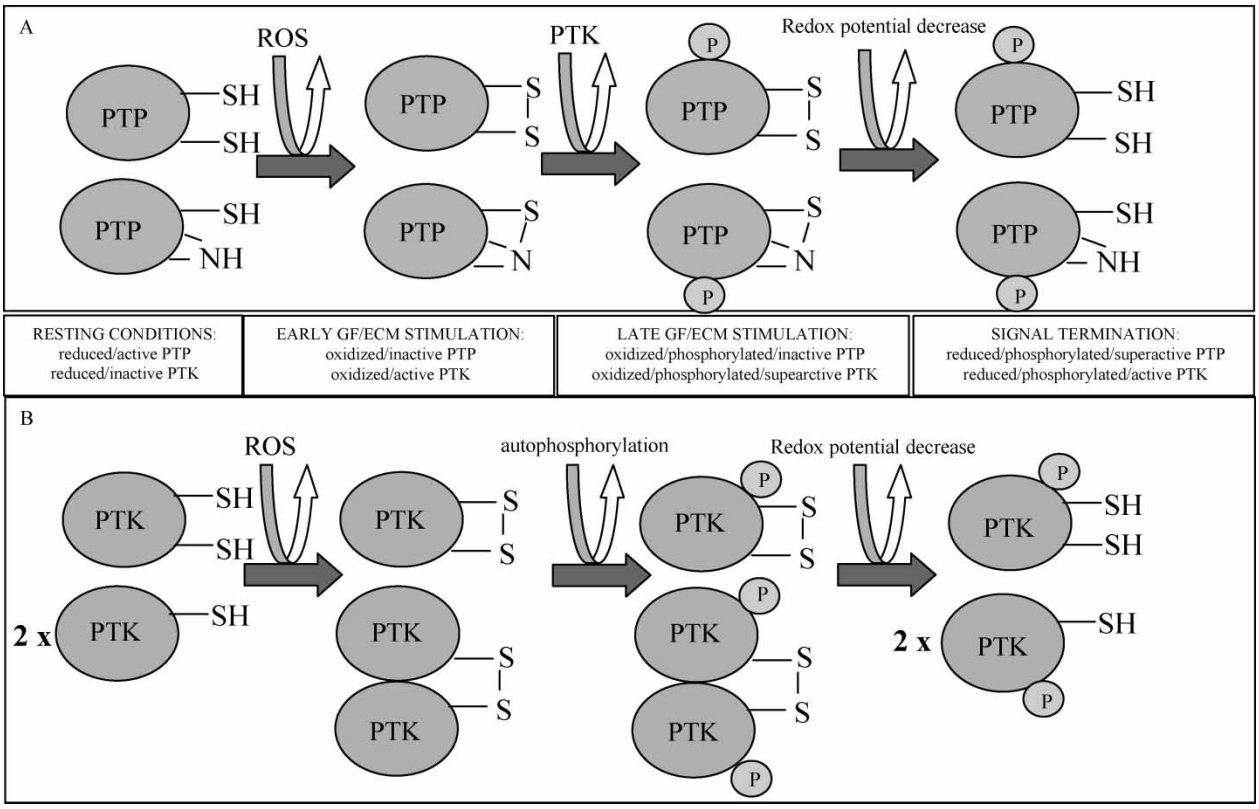

Figure 2. Proposed model of PTP and PTK regulation by oxidation and tyrosine phosphorylation during signal transduction. A: During the early phase of redox-based signalling, i.e. integrin and GF receptors activation, ROS production is able to oxidize both PTPs and PTKs, leading to opposing enzymatic activity regulation, namely inhibition for PTPs and activation for PTKs. Oxidation of PTPs may lead to either the formation of an intramolecular S–S bridge or of a sulphenyl–amide bond. Oxidation of PTKs is more likely linked to the formation of an S–S bond, either intramolecular or between different monomers and leading to dimerization. During this phase oxidized PTPs are concomitantly phosphorylated by cellular PTKs, although this modification is for the moment without consequences due to redox-mediated PTP inactivation. B: In parallel, oxidized and activated PTKs respond with an autophosphorylation in a/several tyrosines, generally acting as further promoter of enzymatic activity and/or as docking sites for SH2 domain containing protein. The complete inactivation of PTPs catalytic activity and the superactivation of PTKs leads as a final event to the propagation of signal transduction. During the second phase of signalling the decrease of intracellular oxidants allows the recovery of the reduced state of PTPs and PTKs. In this phase PTPs are still phosphorylated, owing to their previous inactivation/oxidation. Hence, PTPs become at this time superactive, due to both the recovery of their reduced state and to the phosphorylation-mediated enhancement of their activity. In this condition, PTPs are able to efficiently dephosphorylate their substrates, thus terminating the elicited signal.

The involvement of cysteine oxidation to the activation of RTK is illustrated by cRet. c-Ret is an RTK with a cadherin-like domain in its extracellular region. The c-RET proto-oncogene encodes a receptor-type tyrosine kinase, and its mutations in the germ line are responsible for the inheritance of multiple endocrine neoplasia type 2A and 2B (MEN2A/B). Ret kinases are constitutively activated as a result of MEN2A mutations or MEN2B mutations[77]. The production of ROS induced by the ultraviolet irradiation of cells expressing c-Ret resulted in the dimerization of many c-Ret molecules on cell membrane[78]. Dimerization was mediated by the formation of a disulfide between the Cys residues of adjacent monomer, and the dimerized receptors were preferentially autophosphorylated, resulting in their strong activation in response to ROS production during UV exposure (Figure 2B). In particular ROS contribute to the oncogenic potential of c-Ret as UV light irradiation induces superactivation of the constitutively activated Ret-MEN2A and Ret-MEN2B[78]. It has been suggested that Ret Cys376 is one of critical target amino acids of UV irradiation for Ret kinase activation. Overexpression of superoxide dismutase in cells as a result of gene transfection prevented both the UV-mediated promotion of dimerization and the superactivation of Ret-MEN2A kinase, thereby suggesting that the UV-induced free radicals in cells induce intracellular domains of Ret to dimerize the kinase proteins for superactivation (Figure 2B).

Finally, nitration of Tyr residues in PDGF-R is observed in mild oxidants-treated cells, thus suggesting that ROS-induced modifications can occur at the GF receptor level and can be involved in the regulation of signalling pathways[79]. This redox modification of PDGF-R leads to Src, dependent, activation of ERK and Akt, although being, independent, on PDGF-R tyrosine kinase

activity. In addition, N-ethylmaleimide, which selectively alkylates free thiol groups of cysteine residues, completely inhibited the kinase activity of PDGFR-β. Through site-directed mutagenesis, two conserved cysteine residues critical for the enzymic function of PDGFR-β have been identified: Cys-822, positioned in the catalytic loop, and Cys-940, located in the C-terminal kinase sub domain. Although the non-reducing gel analysis indicated that none of these cysteine residues contributes to the kinase activity by disulphide-bond formation, their role in tyrosine kinase activation is highly feasible as each individual significantly reduced the activities of autophosphorylation and phosphorylation of exogenous substrates[80].

Among intracellular PTKs, the Src tyrosine kinase and some of the members of its family, are reported to be *in vitro* redox regulated. A direct evidence of a redox-linked chemical modification of Src kinase has been obtained from *in vitro* experiments on nitric oxide (NO)-releasing agents [81]. Exposure of Src–NO-releasing agents clearly promoted the catalytic activity of the kinase, either towards its autophosphorylation site or to its downstream substrates. In parallel NO scavengers prevented its activation. The NO-induced Src activation seems to be, independent, from the phosphorylation on Src Tyr527, the autoinhibition site. Correspondingly, a small portion of Src molecules are polymerized through S–S bond formation and NO promotes this polymerization in close association with enzyme activation. The observation that the ROS targeted c-Ret cysteine residue is highly conserved in various non-receptor PTKs, including Abl, Src and Lck, suggests that it might also play a role in the activation of these enzymes. Consistent with this proposal, Src C-terminal residues are crucial for protein stability and cell transformation by this kinase[82]. The relevance of Src redox regulation for anchorage-dependent, growth is documented by our recent data. Src tyrosine kinase undergoes oxidation/activation, likely owing to an S–S bond between Cys245 and Cys487, located in the SH2 and in the kinase domain of the Src molecule, respectively. We demonstrated that the tyrosine kinase c-Src is oxidized in response to cell attachment to ECM and that this modification leads to an enhancement of tyrosine kinase activity and activation of downstream Src-dependent signalling, likely due to the ROS burst following integrin engagement (Chiarugi P., submitted) (Figure 2B).

Indirect regulation of PTKs through redox control of PTPs:

Beside direct oxidation of PTKs, their indirect redox regulation through reversible PTP oxidation in response to GF administration is a well recognised phenomenon[71]. The emerging hypothesis is that the transient negative regulation of PTPs, due to oxidants produced in response to RTK ligand stimulation, represents a strategy adopted by cells to promote RTK signalling by avoiding its prompt inactivation by PTPs (Figure 3). The functional relevance of ROS-mediated PTP inhibition in GF signalling has been demonstrated by blocking their accumulation. The first evidence was provided by Sundaresan, who demonstrated that overexpression of catalase in vascular smooth muscle cells blocked

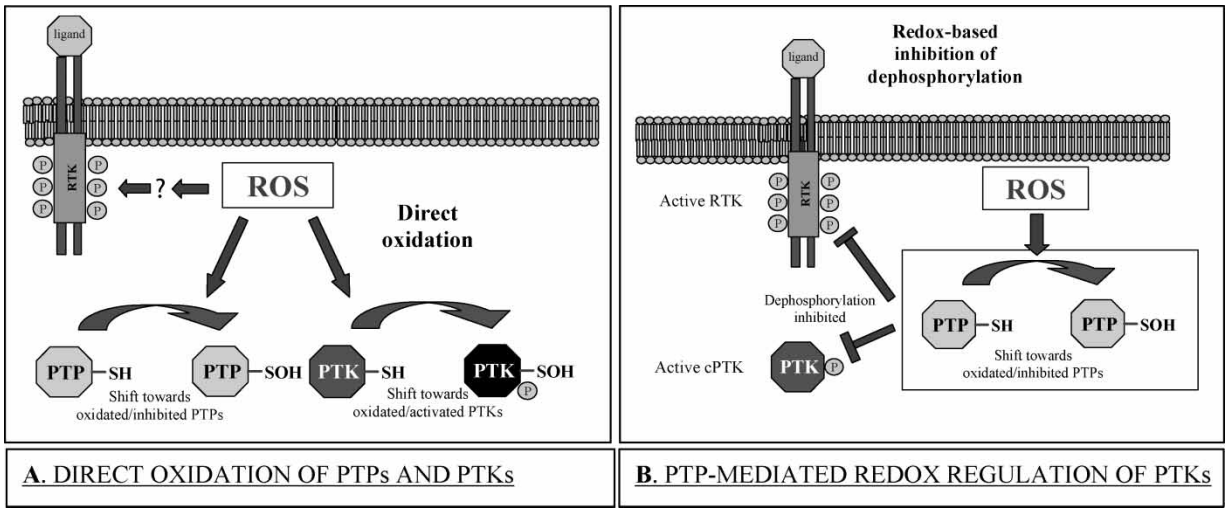

Figure 3. Interplay of redox regulation of PTPs and PTKs. A: Direct oxidation of PTPs and PTKs: PTPs and PTKs respond to direct oxidation in an opposite manner, i.e. with inhibition and activation, respectively. All PTKs in response to activation undergo autophosphorylation in a/some activatory tyrosines/s. B: PTP-mediated redox regulation of PTKs: the redox inactivation of PTPs causes the long-lasting hyperphosphorylation of PTK activatory tyrosine/s, thus causing a further and sustained increase of PTK activity. This could apply to both RTKs and cPTKs, causing, respectively, a sustained GF signalling and a persistent cytoplasmatic tyrosine phosphorylation of cPTK targets.

PDGF-R-induced tyrosine phosphorylation of ERK, as well as PDGF-induced DNA synthesis and migration[50]. Second, interference with H_2O_2 production through catalase loading of A431 cells dramatically reduced tyrosine phosphorylation of EGF-R[49]. Third, catalase pretreatment abolished the insulin-stimulated production of ROS, as well as the inhibition of PTP1B, and was associated with reduced tyrosine phosphorylation of insulin receptor[48]. Finally, the block of ROS production in PDGF stimulated cells leads to the reduction of PDGF-R tyrosine phosphorylation and the kinetics of ROS production. PTP redox inhibition and receptor phosphorylation, thus, display an excellent alignment, suggesting a strict temporal correlation between these events[83]. Hence, it is expected that the redox inhibition of PTPs has an important role in RTK signalling, and that the salvage (*via* re-reduction) of the PTP catalytic activity after oxidation is followed by a dephosphorylation of the activated receptor, thus terminating the signal elicited from the receptor (Figure 3). In this light at least two PTPs have been indicated as responsible for the indirect redox regulation of PDGF-R, namely LMW-PTP [61] and SHP2 [62], while PTP1B and TC45 have been proposed for the same role for the insulin receptor[66].

In agreement with these data on RTKs, cytosolic PTKs are indirectly regulated through reversible inhibition of PTPs (Figure 3). Only few studies demonstrated the effects of ROS on FAK functions. Oxidative stress induces a decrease in the distribution of FAK in focal contacts, without alteration of the integrins in adherent trabecular meshwork[84]. An increase in FAK tyrosine phosphorylation owing to oxidative stress, has been described in different cells in human glioblastoma cell line T98G[85], in human umbilical vein endothelial cells[86], in bovine pulmonary artery endothelial cells[84] and in mesangial cells[87]. More recently the molecular basis of FAK redox regulation has been described by our group. We reported that the endogenous and physiological ROS burst, occurring after integrin receptor engagement, culminated in the inactivation of a FAK phosphatase, namely LMW-PTP[37]. LMW-PTP is oxidized and inhibited in response to integrin engagement and in strict concomitance with the peak of ROS induced by ECM contact. LOX inhibitors, which selectively block integrin-mediated ROS generation, impede LMW-PTP oxidation/inhibition and consequently cause FAK downregulation. The redox regulation of FAK, through the inhibition of its dephosphorylation by LMW-PTP, leads to other key downstream events, including ERK phosphorylation, Src phosphorylation, focal adhesion formation and cell spreading, which are all significantly attenuated by inhibition of redox signalling[37]. It is likely that, in addition to LMW-PTP, other redox sensitive PTPs cooperate to the regulation of the tyrosine phosphorylation of cell adhesion molecules. Although direct evidence are lacking, both SHP2 [88] and PTEN [89] are good candidates for this role, due to their ability to dephosphorylate FAK, thus leading to cytoskeleton rearrangement.

In agreement with these previous findings on FAK, the redox control of the Src tyrosine kinase activity has a PTP-based component. We recently observed that the oxidative burst after integrin engagement leads to Src kinase activation owing to the concomitant action of direct Src oxidation and to PTP inhibition. Although our data cannot quantify the relative contribution to Src redox regulation of direct kinase oxidation or of PTP inhibition, they stress that these two phenomena are concurrent in the achievement of the full activation of the Src kinase during cell/ECM contact (Chiarugi, submitted).

Finally the two modifications that PTPs and PTKs undergo during signalling, namely oxidation and phosphorylation, may influence each other. In particular a given PTP, while oxidized/inhibited, can be efficiently phosphorylated by a PTK, the oxidation being a protection from autodephosphorylation. The phosphorylation of the given PTP, which causes an increase of phosphatase catalytic activity, will then determine a superactivation of the PTP, upon SH reduction. On the contrary a given PTK, while oxidized/activated, can further strengthen its activation through autophosphorylation (Figure 2). In both cases the first modification will prepare for the second one. The available supporting data are very few, likely due to experimental difficulties in the separation of the two phenomena. Remarkably, the oxidation of a given PTP may control its tyrosine phosphorylation state. Meng et al. reported that the oxidized SHP-2 is greatly hyperphosphorylated with respect to the reduced SHP-2, likely due to redox-mediated inactivation of the autocatalytic activity[62]. In agreement, the tyrosine phosphorylation of oxidized PTP1B, in response to IRK activation, was greatly improved[69]. The redox-mediated inhibition of the autocatalytic activity of PTP1B was indicated as responsible of the hyperphosphorylation of the oxidized phosphatase. These hints advocate that tyrosine phosphorylation of an oxidized PTP, i. e. transiently inactive, may be preparatory for the successive phase, i.e. the reduction-mediated recovery of activity. By this way the tyrosine-phosphorylated, newly reduced PTP, may recover an enhanced enzymatic activity, thus being super-active and guaranteeing an efficient termination of signal. A specular situation may be observed during PTK activation, where oxidation may increase the autophosphorylation (and hence activation) of a given PTK (Figure 2). In agreement, both Src and Ret kinases, after being activated through oxidation,

further increase their tyrosine phosphorylation levels by autocatalytic activity[78,81].

The emerging picture point out antagonistic redox regulation of PTPs and PTKs as a key point during important processes such as cell proliferation and adhesion. We can outline a complex redox circuitry whereby, upon ROS increase due to cell adhesion and/or GF stimulation, oxidative inhibition of PTPs, together with direct oxidation of some PTKs leading to their activation, promotes the activation of the downstream outcomes (Figure 3). By this way the antagonistic regulation between PTPs and PTKs, respectively being inhibited and activated by ROS, became really apparent as both these modifications cooperate in achieving a properly executed anchorage-dependent, cell growth.

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