ANTIOXIDANTS & REDOX SIGNALING
Volume 9, Number 1, 2007

Comprehensive Invited Review

Protein Tyrosine Phosphorylation and Reversible Oxidation: Two Cross-Talking Posttranslation Modifications

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Reviewing Editors: Jurgen Bernhagen, Richard Franklin, Gary Schieven, and Masuko Ushio–Fukai

ABSTRACT

In addition to protein phosphorylation, redox-dependent posttranslational modification of proteins is emerging as a key signaling system, conserved throughout evolution, and influencing many aspects of cellular homeostasis. Recent data have provided new insight about the interplay between phosphorylation- and redox-dependent signaling, and reactive oxygen species have been included among intracellular signal transducers of growth factor and extracellular matrix receptors. Both tyrosine phosphorylation and thiol oxidation are reversible and dynamic, and this review will particularly focus on the cross-talk between these posttranslational protein regulatory means. Although these modifications share their reversibility, their effects on enzymatic activity of protein tyrosine phosphatases (PTPs) and protein tyrosine kinases (PTKs) may be even opposite. Indeed, while tyrosine phosphorylation is frequently correlated to enzyme activation, thiol oxidation leads to inactivation of PTPs and to superactivation of PTKs. Several papers describe that both these modifications occur during the same input, (*i.e.,* cell proliferation and motility induced by numerous growth factors and cytokines). The review will discuss several aspects of phosphorylation\oxidation interplay, describing both convergent and divergent features of the integrated and coordinated function of PTPs and PTKs during signaling. *Antioxid. Redox Signal.* 9, 1–24.

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I. INTRODUCTION

PROTEIN TYROSINE PHOSPHORYLATION controls many cellular processes, especially those involved in intercellular communication and coordination of complex functions like cell motility and proliferation. Protein tyrosine phosphorylation is driven by the coordinated action of two superfamilies of proteins called protein tyrosine kinases (PTKs), which are represented by 90 genes in the human genome, and protein tyrosine phosphatases (PTPs) represented by 107 human genes (4, 132).

A. Protein tyrosine kinases

PTKs, also known as tyrosine protein kinases, are usually divided into two families, the transmembrane receptor family and the nonreceptor family (22). Receptor and nonreceptor PTKs are vital enzymes in cellular signaling processes, regulating cell metabolism, growth, migration, and differentiation (3, 88, 98, 206). A recent comprehensive analysis of the human "kinome" identified 518 putative protein kinase genes, of which 71 have not previously been reported or described as kinases (132). In addition, the strict relationship between cancer and a PTK family is revealed through chromosomal mapping, indicating that up to 100 PTKs map to disease loci or cancer amplicons.

Kinase activity of PTKs is closely guarded through autoregulatory mechanisms, as well as by the action of PTPs. Structural studies have revealed several modes of autoregulation for the catalytic activity of these enzymes, and atypical catalytic activity of many PTKs, via mutation or overexpression, has an important function in numerous pathological conditions (22).

Receptor PTKs (RTKs) are cell-surface, transmembrane receptors carrying a multidomain extracellular segment binding polypeptide ligands, a single-pass transmembrane helix, and a cytoplasmic segment containing a tyrosine kinase domain together with several regulatory sequences located both N- and C-terminal to the kinase domain (96). The RTK family includes, among others, epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), and the insulin receptor kinase (IRK). Conversely, about one-third of tyrosine kinases are classified as nonreceptor cytoplasmic tyrosine kinases (cPTKs). They lack a transmembrane section, and generally function downstream of membrane bound receptors (Fig. 1). These can be divided into nine canonical families: Abl, Fes/Fer, Syk/Zap70, Jak, Tec, FAK, Ack, Src, and Csk,

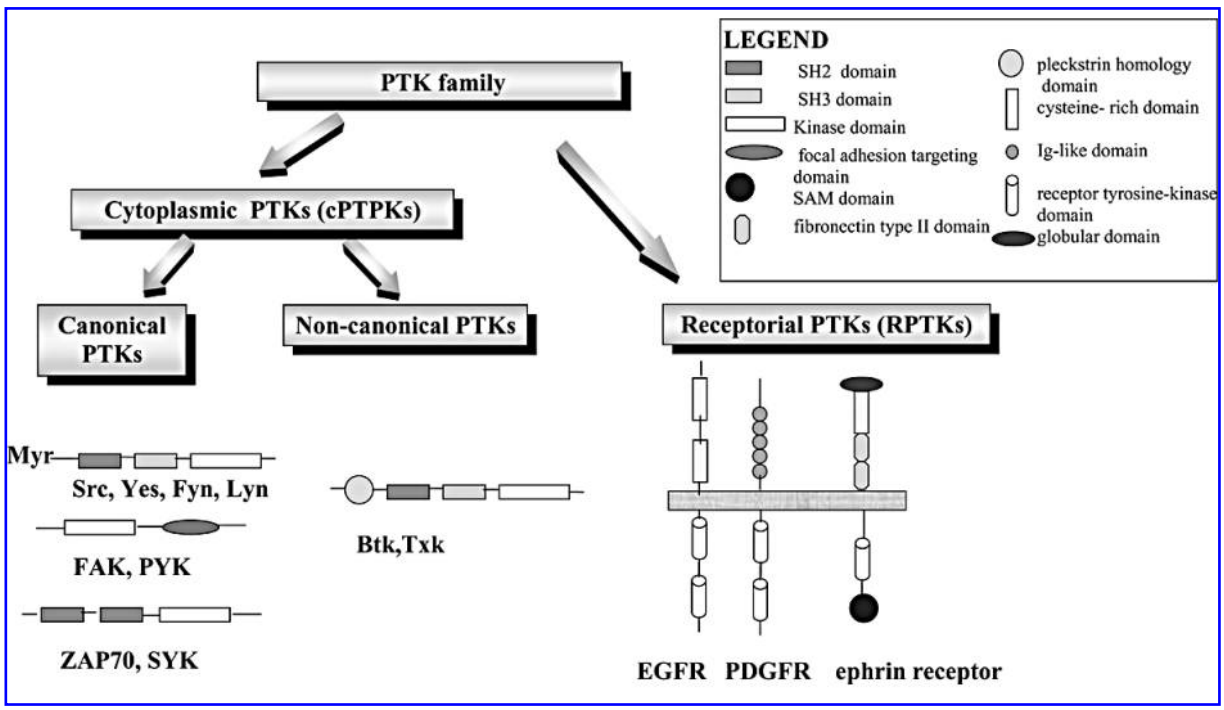

FIG. 1. PTK family. PTKs are commonly divided into a larger subfamily of membrane receptor (RTKs) and a smaller subgroup of cytosolic proteins (cPTKs). RTKs are cell-surface, transmembrane receptors carrying a multidomain extracellular segment binding polypeptide ligands, a single-pass transmembrane helix, and a cytoplasmic portion containing single or multiple tyrosine kinase domains jointly with several regulatory sequences, located both N- and C-terminal to the kinase domain (96). cPTKs represent about one-third of tyrosine kinases. They lack a transmembrane section, and generally act downstream of membrane receptors. cPTKs have been divided into nine canonical families: Abl, Fes/Fer, Syk/Zap70, Jak, Tec, FAK, Ack, Src, and Csk and four noncanonical cPTKs, namely Rlk/Txk, Srm, Rak/Frk, and Brk/Sik. For guaranteeing appropriate subcellular localization and/or proper autoinhibition, cPTKs typically contain modular domains that provoke protein–protein or protein–lipid interactions. In addition, the Src subfamily members are frequently myristoylated.

and four noncanonical cPTKs, namely Rlk/Txk, Srm, Rak/Frk, and Brk/Sik (148). For appropriate subcellular localization, cPTKs typically contain modular domains that provoke protein–protein (*e.g.*, Src homology, SH2/SH3 domains) or protein–lipid interactions (*e.g.*, pleckstrin homology [PH] domain) (161), and are frequently lipid-modified (*e.g.*, myristoylated) (23). In general, tyrosine autophosphorylation greatly stimulates the catalytic activity of PTKs, and furthermore generates some docking sites for recruitment of downstream substrate proteins (95, 165, 235).

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 noncontiguous 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 (96).

Most PTKs are maintained in a low activity state through a variety of autoregulatory mechanisms, all of which avoid the most favorable configuration of the kinase active site. Even though the conformation of the catalytic loop is outstandingly comparable among PTKs, and between inactive and active states, other segments, in particular α-helix C and the activation loop, are often switch elements in intramolecular regulation.

In general, activation of RTKs is achieved through ligand binding to the extracellular domain, which stabilizes a dimeric receptor arrangement, facilitating *trans*-phosphorylation in the cytoplasmic domain (103). Evidence indicates that RTK dimerization *per se* is not enough for kinase activation. An extra prerequisite for ligand-induced activating conformational switches has been indicated, guaranteeing that the catalytic domains are put side by side in an appropriate arrangement to allow phosphorylation in *trans* involving both receptor subunits (103, 178). For some RTKs, including PDGFR, Kit/stem cell factor receptor, colony-stimulating factor-1 receptor, ephrin receptor subfamily, and insulin receptor, the juxtamembrane segment has been implicated in autoinhibition. Consequently, autophosphorylation of one or two conserved tyrosines within the juxtamembrane domain of these receptors, is required for complete kinase activation, and mutation to phenylalanine significantly reduces ligand-induced kinase activation (18).

The activation mechanism of cPTKs is more complex than for RTKs, involving heterologous protein–protein interactions, as well as protein clustering to enable *trans*-phosphorylation. The Src family kinases are the prototypical nonreceptor cPTKs, and their structure has been studied extensively (186). In all members of the Src family, namely Src, Fyn, Lyn, Yes, and Lck, the SH2 domain interacts with phosphorylated Tyr 527 in the C terminus and the SH3 domain with the polyproline helix in the region next to the SH2 domain. Phosphorylation of Tyr 527 is mediated by C-terminal Src kinase (CSK) and CSK-homologous kinase (CHK). CSK and CHK are mainly cytosolic while Src family kinases are anchored to the plasma membrane. CSK and CHK recruitment to the plasma membrane is mediated by the interaction of their SH2/SH3 and/or kinase domains to specific transmembrane proteins, adaptor proteins, or G-proteins. CSK interaction with Src family kinases is transient while CHK binds firmly to form stable protein complexes with Src family kinases (40). The strong intramolecular interactions between the SH2 domain and the C-terminal tail, and between the SH3 domain and the SH2–kinase linker, avoid the kinase domain from assuming an active configuration (223). This causes misalignment of residues that are essential for kinase activity. Besides, 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. Candidate phosphotyrosine 527 phosphatases include cytoplasmic Src homology phosphotyrosine phosphatase1 and 2 (SHP1 and SHP2), PTP1B, and transmembrane enzymes as CD45, PTPα, PTPϵ, and PTPλ. Conversely, dephosphorylation of phosphotyrosine 416 decreases Src kinase activity. Thus far PTP-BL, the mouse homologue of human PTP-BAS, has been shown to dephosphorylate phosphotyrosine 416 in a regulatory fashion (46).

Besides SH2/SH3 and autophosphorylation global switches, several redox-linked signal transduction pathways have been recently proposed as concurrent mechanisms for PTK activation (see below).

B. Protein tyrosine phosphatases

PTPs can exert both positive and negative effects on signaling pathways and play vital physiological roles in a multiplicity of mammalian cells (4, 6, 27, 28, 146, 159, 177, 178, 194, 217). As reported for PTKs, deregulation of PTP activity can strongly contribute to the pathogenesis of many human diseases, ranging from cancer to cardiovascular, immunological, infectious, neurological, and metabolic diseases (9, 31, 64, 76, 154, 217, 229, 229). For example, many PTPs, including LAR, TC-PTP, LMW-PTP, and SHP2, have been indicated as therapeutic targets for human diabetes, although the proposed mechanisms vary for the different PTPs from the regulation of insulin receptor signaling to the control of insulin resistance (25, 64). In addition, several PTPs have been involved in cancer onset and progression (153). PTPs can either function as tumor suppressors, or conversely as oncogenes. The tumor-suppressive function of PTPs is indicated by repeated inactivating mutations of PTPs in colon cancer, and the discovery of *Ptprj* as a colon cancer susceptibility gene in STS/A mice. Moreover, inactivation of the SHP1 gene by methylation has been described in hematological malignancies. Conversely, a PTP oncogenic activity has been indicated only for the mutational activation of SHP2, which occurs in hereditary and sporadic leukemias and, less frequently, in solid tumors (153).

PTPs are a family of enzymes whose structural multiplicity and complexity rival those of PTKs. However, dissimilar from PTKs, which share sequence homology with protein serine/threonine kinases, PTPs exhibit no sequence correspondence with the protein serine/threonine phosphatase family. All PTPs share a common CX_5R active site motif and an identical catalytic mechanism involving a catalytic cys-

teine and an aspartic acid. In addition to the catalytic domain, PTPs may possess a wide range of structural elements, including SH2 domains, PDZ domains, extracellular ligand binding domains, and many others (217, 230). A recent evaluation from the nearly completed human genome sequence suggested that humans have 107 PTPs (4), including both the tyrosine-specific and dual-specific phosphatases. The tyrosine-specific phosphatases, such as PTP1B, act on phosphotyrosine-containing proteins, whereas the dual-specific phosphatases, such as Cdc25, can exploit protein substrates containing phosphotyrosine, as well as phosphoserine and phosphothreonine (5). The tyrosine-specific PTPs should be divided into two groups: receptor-like (RPTPs) and cytosolic PTPs (cPTPs) (Fig. 2). RPTPs, exemplified by R-PTPα and CD45, generally have an extracellular ligand-binding domain, a single transmembrane region, and one or two cytoplasmic catalytic domains. The intracellular PTPs, exemplified by PTP1B, low molecular weight-PTP (LMW-PTP), and SHP2, contain a single catalytic domain and various amino or carboxyl terminal extensions. They may contain SH2 domains that have targeting or regulatory functions. Examples of dual-specificity phosphatases embrace the mitogen activated protein kinase (MAPK) phosphatases, the cell cycle regulators Cdc25 phosphatases, and the tumor suppressor phosphatase and tensin homologue deleted on chromosome ten (PTEN), that is also a critical lipid phosphatase (4, 230). All PTPs are characterized by: (a) their sensitivity to vanadate, (b) ability to hydrolyze *p*-nitrophenyl phosphate, (c) insensitivity to okadaic acid, and (d) lack of metal ion requirement for catalysis (57).

The active site of PTPs is positioned inside a crevice on the protein outside. The much deeper active site pocket in the tyrosine-specific phosphatases (9 Å) selects solely pTyr-containing substrates (102), whereas the more superficial active site crevice for the dual-specificity phosphatases (6 Å) may contain both pTyr and pSer/pThr (193, 226). PTP enzymes

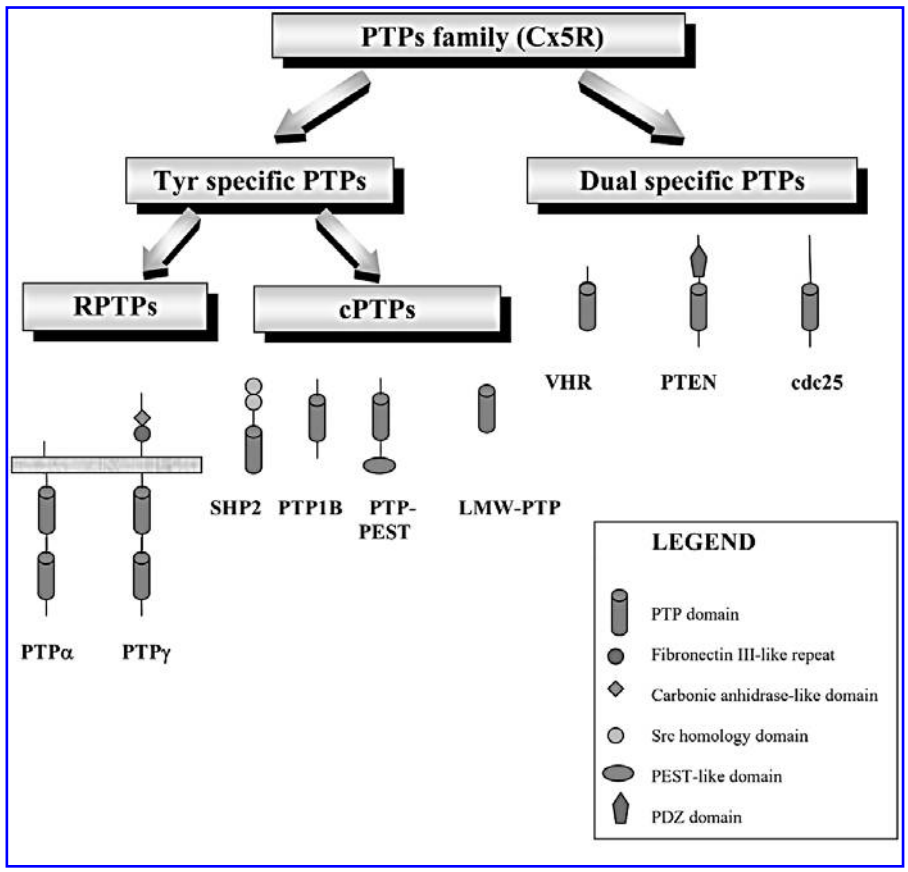

FIG. 2. PTP family. PTPs, although they all share a common CX_5R active site motif and an identical catalytic mechanism, are a very heterogeneous class of enzymes. They may be divided into tyrosine-specific and dual-specific phosphatases. The tyrosine-specific phosphatases act on phosphotyrosine-containing proteins, whereas the dual-specific phosphatases can dephosphorylate protein substrates containing phosphotyrosine, as well as phosphoserine and phosphothreonine. The dual-specificity phosphatase group is the most heterogeneous: it embraces the MAPK phosphatases, the cell cycle regulators Cdc25 phosphatases, and the tumor suppressor PTEN, which is able to dephosphorylate both protein and lipid substrates. The tyrosine-specific PTPs can be further considered into two groups: receptor-like (RPTPs) and cytosolic PTPs (cPTPs). RPTPs generally have an extracellular ligand-binding domain, a single transmembrane region, and one or two cytoplasmic catalytic domains. The intracellular PTPs contain a single catalytic domain and several kinds of regulatory amino or carboxyl terminal extensions (SH2 domains for targeting and\or regulatory functions, PEST domain for proteolytic control, phosphorylation sites for protein/protein interaction, and enzymatic activity regulation, etc.)

use the thiol group of the active site cysteine as the attacking nucleophile to form a cysteinyl-phosphate enzyme intermediate (E-P) (41, 85). Mutagenesis-directed substitutions of the Cys residues completely abolish PTP activity. The nucleophilic cysteine is allocated within the active site, specifically designed to bind a negatively-charged substrate. Thereafter, catalysis is supported by a conserved aspartic acid and arginine residues (41, 231).

Unlike most cysteine residues within proteins, which remain protonated at physiological pH, showing a p*K*a of about 8.0, the PTP catalytic site cysteine residue is extremely reactive and rapidly forms a thiolate anion at physiological pH, suggesting that their *in vivo* p*K*a actually is around 6. The reactivity of this SH group is indeed essential for the catalytic mechanism of all PTPs, which requires a phospho-Cys intermediate. On the other hand, the exceptional reactivity required for enzymatic activity in turn renders the catalytic cysteine susceptible to oxidation. *In vitro* studies have suggested that mild hydrogen peroxide treatments readily inactivate several PTPs, while having no effect on serine/threonine phosphatases (58). Hydrogen peroxide treatment of several PTPs resulted in the reversible formation of a sulfenic acid (Cys-SOH) intermediate, further stabilized by disulfide or sulfanilamide bonds (34).

RPTPs are mainly negatively regulated through dimerization, a known regulatory mechanism for many signal transduction molecules, in particular, transmembrane receptor proteins (20). Widespread studies have established that ligand-induced dimerization is decisive for the activation of RTKs, antigen receptors, and cytokine receptors. Frequently, dimerization activates receptor kinase activity, either endogenous or associated, by *trans*-autophosphorylation (160). Conversely to RTKs, RPTPs are often inhibited by dimerization, but in most cases, the ligands that induce such a regulation have not been identified (20). The current model for negative regulation of RPTPs has been elucidated for both CD45 and receptor protein-tyrosine phosphatase-α (RPTPα). In their monomeric state, CD45 or RPTPα are active and activate a Src Family Kinase (Lck or Src) by dephosphorylating their inhibitory tyrosine. Dimerization of the RPTP causes its inactivation through the symmetrical interactions between the phosphatase catalytic site and the inhibitory structural block (55).

cPTPs display a more composite 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 (147). As for numerous SH2-domain containing enzymes, being catalytically inactive owing to SH2 domain-mediated autoinhibition, these PTPs are essentially inactive under basal conditions. Actually, during quiescent conditions the SH2 domain of these PTPs is inserted into the catalytic cleft, thus resulting in autoinhibition of the PTP domain. The displacement of autoinhibiting SH2 domain of SHP-2 by tyrosine phosphorylated ligands dramatically increases PTP activity, due to opening of the enzyme structure (13). Of note, both SHPs undergo phosphorylation at both C-terminal tyrosyl residues in response to some growth factors (8). 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 through their own phosphorylation, respectively in Tyr66 for PTP1B and Tyr131 for LMW-PTP (26, 123). Autodephosphorylation has been reported for several tyr-phosphorylated phosphatases, as SHP-2(137), RPTPα (56), SHP-1 (232), and LMW-PTP (80), although the role of *in vivo* autodephosphorylation of PTP is an almost unaddressed question, but this mechanism may add a further level of regulation to cytoplasmic PTPs.

Although RPTPs and cytoplasmic PTPs exhibit different mechanisms of regulation, being both dimerization and tyrosyl-phosphorylation highly specific for each group, they share a common and additional regulatory mechanism: the reversible oxidation of their catalytic cysteine, leading to inhibition of phosphatase activity (see below).

C. Reactive oxygen species and their sources

Reactive oxygen species (ROS) have been recently recognized as important mediators of cell growth, adhesion, differentiation, senescence, and apoptosis. Proteins with low-pK_a cysteine residues, which are susceptible to oxidation by ROS, include several transcription factors as the nuclear factor κ-B (182), activator protein-1 (152), hypoxia-inducible factor (215), p53 (167), the p21Ras family of proto-oncogenes (117), and, of note, the two family of enzymes on which this review is focusing, PTPs and PTKs.

The term ROS covers a range of partially reduced metabolites of oxygen (*e.g.*, superoxide anions, hydrogen peroxide, and hydroxyl radicals) possessing higher reactivity than molecular oxygen. Inside cells they are by-products of normal aerobic metabolism, or second messengers in various signal transduction pathways, including growth factors and integrin signaling (37). Following the early descriptions that certain ligands activate ROS production, the question remained as to whether this event represents only a toxic and nonspecific response, or whether the generation of ROS represents an important element within signaling pathways. Relatively little is known regarding the sources of intracellularly generated that may be regulated in a ligand-dependent fashion. We thereafter focus our report on the available data about the precise way in which ligands could activate ROS production.

First, superoxide anions are a by-product of mitochondrial electron chain flux, accounting for ~2% of total oxygen utilization by the organelle (29), owing to the physiological activity of the respiratory chain, which leads to the production of semiquinones, a potential source of ROS (75). The respiratory chain produces ROS at complex I (NADH/ubiquinone oxidoreductase) and complex III (ubiquinol/cytochrome c oxido-reductase) (Fig. 3). Recently a signaling role for the mitochondrial source of ROS has been suggested both in cancer invasion and in hypoxia signaling (44, 62, 166, 219). In addition, we have evidence that mitochondrial ROS exert a signaling role during integrin-mediated cell adhesion (L. Taddei, personal communication). Additional work is required to confirm a direct activation of mitochondrial complex I and III by these ligand-dependent signaling.

Superoxide anions may also be produced by cytoplasmic enzymes such as NADPH oxidase (NOX), dual oxidases (DUOX), cyclooxygenase, and lipoxygenase during prostanoid metabolism, xanthine oxidase, catecholamine auto-

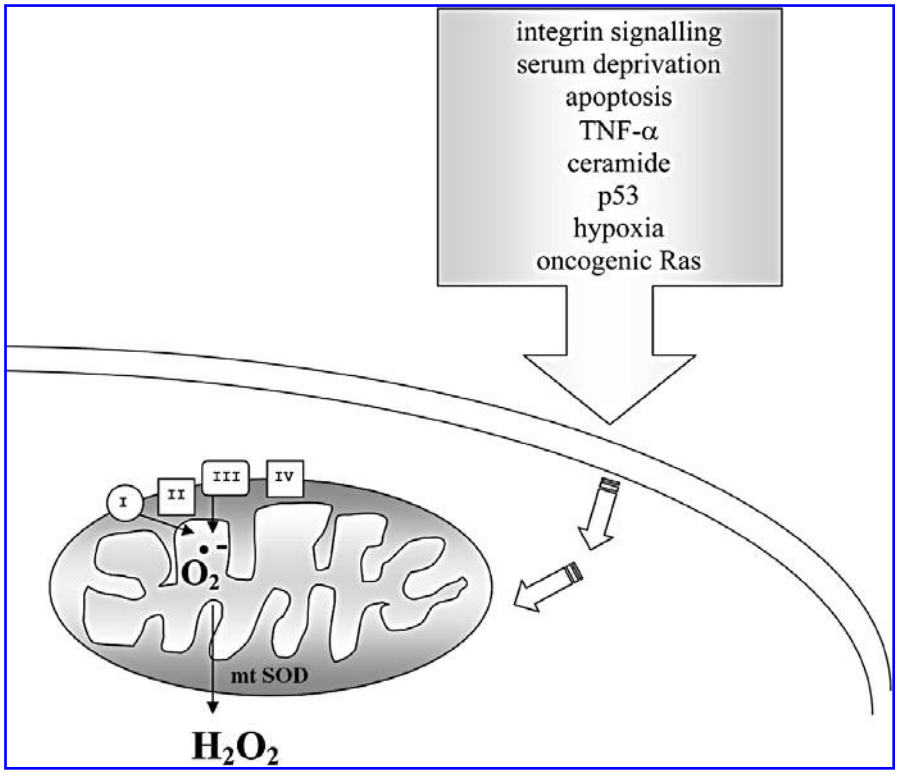

FIG. 3. Generation of ROS by mitochondria. The mitochondrial generation of ROS represents a relevant by-product of electron flow through the respiratory chain (complexes of the respiratory chain are indicated). Electrons from complex I and III can convert oxygen to superoxide ion ($O_2^{\bullet -}$), which is then converted to H_2O_2 by spontaneous or enzymatic dismutation mediated by superoxide dismutase (mitochondrial isoenzyme, mtSOD). Mitochondrial ROS production is itself modulated by a variety of stimuli listed in the box (155), and, at least for integrin signaling, is reported to be Rac dependent (219).

oxidation, and the NO synthases. Although it remained unclear for many years whether nonphagocytic cells contain an NADPH oxidase system comparable to a phagocytic one, numerous types of NADPH oxidases have been identified in fibroblasts and other nonphagocytic cells (54, 196).

NADPH oxidase is a protein complex formed by membrane (gp91phox, p22phox) and cytosolic (Rac, p67phox, p47phox, p40phox) proteins (10) (Fig. 4). The enzyme activity of gp91phox is regulated by the assembly of these regulatory subunits to form an active complex. NADPH oxidase catalyzes the one-electron reduction of O_2 to $O_2^{\bullet -}$, which spontaneously or enzymatically dismutates to H_2O_2. Several lines of evidence reveal that NADPH oxidase is specifically involved in the generation of ROS by soluble GFs, such as transforming growth factor-β1 (150, 203), interleukin-1 (135), tumor necrosis factor-α (125), insulin (129), PDGF and EGF (11, 197), angiotensin II (83), thrombin and lysophosphatidic acid (32).

Membrane oxidases similar to the phagocytic NADPH oxidase complex are expressed almost universally in nonphagocytic cell types (134) and, although sharing different degrees of sequence similarity, they possess the same or similar domain organization. They can be divided into three groups: the first one including NOX1, NOX3, and NOX4, comprises those enzymes that are similar to phagocytic gp91phox for their structure and enzymatic activity; the second group consists of NOX5, which can be activated by calcium because of its calmodulin-like domain; the third group, the DUOX enzymes, besides the calmodulin-like domain, contains an amino-terminal peroxidase-homology domain localized on the extracellular portion of the plasma membrane. The overexpression of the peroxidase domain in *Escherichia coli*, causes H2O2-dependent peroxidative reactions including tyrosine crosslinking. Indeed, DUOX appears to exert a dual function: first generating hydrogen peroxide, and thereafter using it through its own peroxidase domain. This atypical dual function seems appropriate to oxidize extracellular substrates such as extracellular matrix proteins. According to Singh *et al*., DUOX enzymes play a role in a mechanism of positive feed-back, that involves PTPs oxidation and leads to enhancement of B cell receptor signaling (188).

5-Lipoxygenase (LOX) is a mixed function oxidase involved in the synthesis of leukotrienes from arachidonic acid, and its activation, which is regulated by Rac1, is usually followed by translocation to the nuclear envelope (185). LOX converts arachidonic acid into hydroperoxyeicosatetraenoaic acid that is afterwards transformed into leukotrienes, producing also O_2^- that forms H_2O_2 by dismutation. ROS produced by LOX activation have been implicated in several signaling pathways, including growth, contact inhibition, angiogenesis, and integrin-mediated cell adhesion and spreading (Fig. 5) (37, 42, 62).

Recent data point to an extracellular source of hydrogen peroxide in GF signaling. These authors (60) propose that after EGF treatment, hydrogen peroxide could be generated extracellularly by the receptor-ligand interaction and that the

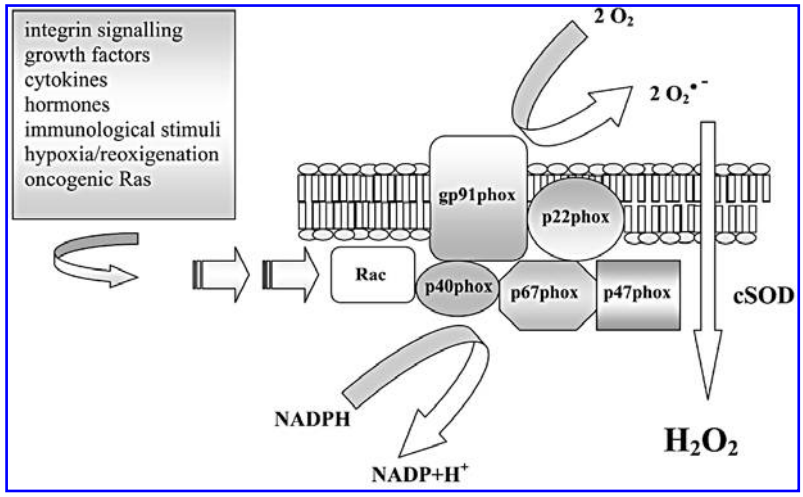

FIG. 4. Generation of ROS by NADPH oxidase. NADPH oxidase is a protein complex formed by membrane (gp91phox, p22phox) and cytosolic (Rac, p67phox, p47phox, p40phox) proteins. The enzyme activity of gp91phox is regulated by the assembly of these regulatory subunits to form an active complex. In response to the activation of the small GTPase Rac, a component of NADPH oxidase, namely p47phox, translocates to the membrane. The membrane oxidase produces $O_2^{\bullet -}$, which is then converted to H_2O_2 again by spontaneous or enzymatic dismutation mediated by superoxide dismutase (cytosolic isoenzyme, cSOD). Membrane oxidases strictly related to the phagocytic NADPH oxidase complex are expressed in nonphagocytic cell types (134). Several evidence reveal that NADPH oxidase is specifically involved in the generation of ROS by soluble GFs, cytokines, and other stimuli listed in the box (1, 48, 93, 112, 125, 195).

oxidant molecules, flowed inside the cells, are involved in EGF signal transduction. Besides the novelty of these data on EGF signaling, the generality of the extracellular generation of hydrogen peroxide in response to receptor ligation still has to be proven.

Despite the many findings describing the regulation by extracellular stimuli of hydrogen peroxide production, little is known about how hydrogen peroxide is really distributed to the cytoplasm. NADPH oxidase releases hydrogen peroxide that has in turn to be imported inside the cell. Both NADPH oxidase and LOX have been detected in the endoplasmic reticulum and nucleus (114, 163, 209) and can alternatively deliver the produced hydrogen peroxide into the luminal space of such organelles. In any case, hydrogen peroxide must cross the membrane to act on intracellular redox-sensitive targets, although the mechanism is fully unknown. Recent findings indicate that many membranes are almost impermeable to hydrogen peroxide as well as to water (7, 130, 183, 192). It is likely that, in keeping with the transport of water for which a key role of aquaporins has been reported (77, 90, 101, 124, 126, 138, 192, 205, 207), hydrogen peroxide does not simply diffuse across cellular membranes but needs particular transporters or diffusion-facilitating proteins (17). These molecules have not yet been definitely identified, although Bienert finds that aquaporin8 and aquaporinTIP1 leads to a 10-fold increase in hydrogen peroxide sensitivity (17). In addition to aquaporins, a role for the lipid composition of plasma membrane has been recently proposed for diffusion across the membrane by hydrogen peroxide (69, 192). We can therefore speculate that aquaporin regulation and/or lipid composition of cell membranes could add a further level of regulation to redox signaling.

Finally, microsomes and peroxisomes constitute additional sources of ROS, mainly in stress situations. In particular, prolonged starvation leads to peroxisomes-derived ROS production in consequence of intense fatty acid oxidation. In agreement during hyperoxia conditions, 80% of H2O2 production can be attributed to microsomes (86, 168).

D. Antioxidant defenses

Transitory 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 protection systems have evolved to combat the growth of ROS. These include various nonenzymatic

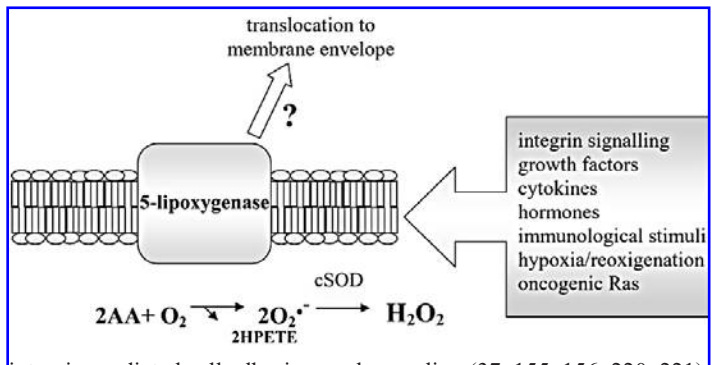

FIG. 5. Generation of ROS by 5-lipoxygenase. 5-Lipoxygenase (5-LOX) is a mixed function oxidase implicated in the synthesis of leukotrienes from arachidonic acid (AA), and its activation, again under the control of the small GTPase Rac1, is usually followed by translocation to the nuclear envelope (12). The products of the reaction catalyzed by 5-LOX are hydroperoxyeicosatetraenoaic acid (HPETE), thereafter converted into leukotrienes, and $O_2^{\bullet -}$, converted to H_2O_2 again by enzymatic dismutation mediated by superoxide dismutase (cytosolic isoenzyme, cSOD). ROS produced by 5-LOX activation have been implicated in several signaling pathways indicated in the box, including growth, contact inhibition, angiogenesis, integrin-mediated cell adhesion, and spreading (37, 155, 156, 220, 221).

molecules (*e.g.*, glutathione, vitamins A, C, and E, and flavonoids) as well as enzymatic scavengers of ROS [*e.g.*, superoxide dismutases, peroxiredoxins (PRX), glutaredoxins (GRX), catalase, and glutathione peroxidase].

The removal of H_2O_2 in cells is mediated predominantly by catalase, glutathione peroxidase, and PRXs. Catalase is localized entirely in peroxisomes, thus elimination of cytosolic H_2O_2 by catalase requires its distribution into these organelles. Glutathione peroxidase is largely restricted to the cytosol but is also present in mitochondria. PRXs, subdivided into two subclasses named Prx I and Prx II, are abundant in the cytosol and exhibit higher affinity toward H_2O_2 with respect to catalase and glutathione peroxidase (171). Consequently Prx I and Prx II are good candidates for ROS scavenging and they have been recently proposed as key regulators of H_2O_2 signaling initiated by cell-surface receptors (171). Indeed, overexpressed or depleted peroxiredoxins affect the intracellular level of H_2O_2 produced in the cells stimulated with PDGF or tumor necrosis factor-α and are able to regulate the signaling elicited by those ligands, indicating that these enzymes are included among signaling molecules involved in redox cascades (105, 106, 115, 211, 228).

Unfortunately, these defense 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 (15, 45, 72, 204, 213). Of note, some of these enzymes have been included among redox-sensitive signaling enzymes and cooperate in the regulation of intracellular protein tyrosine phosphorylation through redox-based mechanisms (39, 115).

Besides their role of ROS scavengers, several enzymatic and nonenzymatic systems can directly protect proteins from oxidation or regenerate oxidized proteins. To regenerate *S*-thiolated proteins, these systems act as reducing agents, rather than as free radical scavengers. In the framework of redox regulation, these systems are key regulatory enzymes and can be compared with the role played by protein kinases and phosphatases in the regulation of protein tyrosine phosphorylation (78). The enzymes that catalyze protein thiol disulfide redox reactions include GRX, thioredoxin (TRX), and protein disulfide-isomerase, and have a CXXC motif in the active site. Oxidoreduction of these cysteines, by either mono- or di-thiol mechanisms, is part of their catalytic cycle (78). TRX is able to drive reduction of sulfenic acids (SOH) and disulfides (S–S), either intermolecular or intramolecular (92). GRX can support the reversibility of mixed disulfides with GSH and *S*-nitrosothiols (SNO), while only sulfiredoxin (SRX) can reverse the formation of sulfinic acids (SO_2H) (19, 222). Glutathionylation of proteins, which occurs through the formation of a mixed disulfide between one cysteine of glutathione and one cysteine of the other protein, constitutes an efficient mechanism to protect proteins from irreversible modifications, and also seems to play an important role in cell signaling. An exiguous number of proteins are recognized to be involved in the glutathionylation process, such as GRX and protein disulfide isomerase, and even if many glutathionylated proteins have been identified to date (PTP1B, *c*-jun, MAPK kinase 1, and β-actin, to mention a few (14, 47, 108, 216), the functional significance of this modification is far to be clearly understood. As many other posttranslational modifications, glutathionylation is reversible and is mediated by SRX1 and GRX (70). Finally, sulfonic acids (SO_3H) are currently believed to be irreversible terminal products of overoxidation.

II. THE INTERPLAY BETWEEN PROTEIN TYROSINE PHOSPHORYLATION AND PROTEIN REDOX MODIFICATIONS

It has been reported that ROS such as H_2O_2 induce tyrosine phosphorylation of cellular proteins, which is strongly potentiated by a combination treatment with vanadate. The mechanism was believed to be attributed to the inhibition of tyrosine phosphatase, or activation of tyrosine kinase, or both. It has been reported that ROS such as H2O2 induces tyrosine phosphorylation of cellular proteins, which is strongly potentiated by a combination treatment with vanadate. Vanadate and pervanadate (the complexes of vanadate with hydrogen peroxide) are two commonly used general protein-tyrosine phosphatase (PTP) inhibitors. These compounds also have insulin-mimetic properties, an observation that has generated a great deal of interest and study. While vanadate is a simple competitive inhibitor for all PTPs, the mechanism by which PTPs are inactivated by pervanadate appears to involve directional oxidation of the essential cysteine residue in the catalytic cleft of the PTP (99).

The tyrosine phosphorylation level of a given protein is the result of the activity of the kinase, which causes its phosphorylation balanced by the activity of the phosphatase affecting its dephosphorylation. This kind of regulation has been extensively studied for several RTKs. The proposed model suggests that both ligand stimulation (increasing kinase activity of RTKs) and redox downregulation of RTK-associated PTPs (decreasing RTK dephosphorylation) guide the accumulation of phosphorylated/activated RTKs, thus leading to signal propagation (Fig. 6).

A. Redox downregulation of PTPs through oxidation and increase in tyrosine phosphorylation

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 (49, 58, 119). 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 studies have provided insight into how PTPs might transduce oxidative stress conditions, leading to a perfect redox regulation of this 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 (119). Subsequent studies demonstrated that in the presence of glutathione the cysteine-sulfenic intermediate formed by oxi-

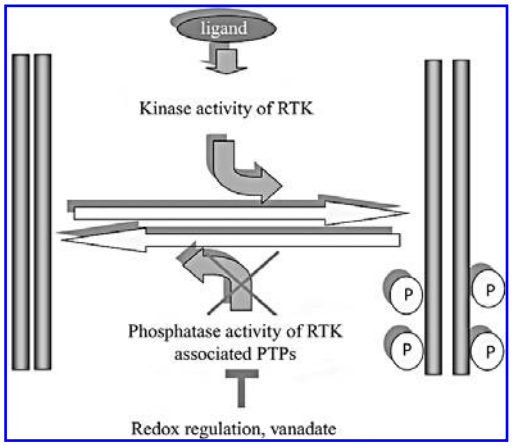

FIG. 6. Activation of RTKs. The phosphorylation level of a given RTK is the result of its intrinsic tyrosine kinase activity and of the dephosphorylation degree of its phosphorylated tyrosines by receptor-associated PTPs. The shift toward phosphorylation of RTKs, associated with its activation and downstream signaling, can be achieved either by ligand binding enhancement of kinase activity, or by inhibition of RTK-associated PTPs. The latter has been reported to occur by vanadate treatment or by redox downregulation of these enzymes, either transiently (during signaling) or everlastingly (during oxidative stress conditions).

dation of PTP1B could form a mixed disulfide (14), thus emphasizing that protein glutathionylation may be a physiological redox-based protein modification (94). A reversible oxidation was then demonstrated for LMW-PTP throughout PDGF stimulation (30, 36). 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 been described by Meng *et al*. using a functional modified in gel-PTP assay (137). More recently, SHP2 oxidation has been reported during T cell receptor signaling and lymphocyte adhesion (116) and during integrin–mediated cell adhesion of fibroblasts and cell spreading (L. Taddei, personal communication). Besides, Rhee and colleagues point up intramolecular S–S bridge-dependent *in vitro* redox regulation of lipid phosphatase PTEN (120), while Savintsky provides evidence for Cdc25C phosphatase degradation triggered by H_2O_2-induced disulfide bond formation (175). In addition, H_2O_2-dependent oxidation stabilizes dimers of transmembrane RPTP-α, resulting again in phosphatase inactivation (21), by inducing intermolecular disulfide bond formation. 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 spliced variant of the T cell protein-tyrosine phosphatase (136).

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. As outlined above, different cysteine residues can take part to the disulfide bridge during PTP oxidation. For example, cysteine disulfide may derive from two cysteines within the catalytic site as for LMW-PTP (30, 36), between distant cysteines as for Cdc25 (175), RPTPα (21), and PTEN (121), as well as between PTPs and glutathione in the case of PTP1B (14). 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 (173, 210). 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 cystine or by GSH mixed disulfide formation) or by transient S–N sulfenic-amide bond (34, 38) (Fig. 7).

As not yet identified, intracellular reducing agents such as TRX, GRX, and reduced glutathione are thought to play es-

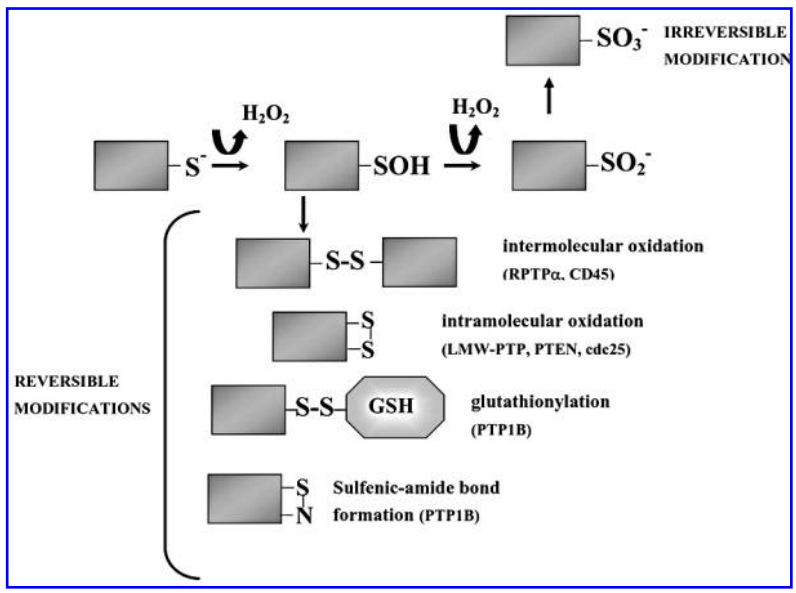

FIG. 7. Redox modification of protein sulfhydryls. **S**cheme of reversible and irreversible modifications for redox sensitive proteins. A reactive thiolate is oxidized to sulfenic acid by low doses of hydrogen peroxide. This specie can be further oxidized to sulfinic acid or sulfonic derivates, which are irreversible by products sent to protein degradation. Conversely, the reversibility of redox modifications during signaling is guaranteed by the formation of a disulfide or a sulfenyl–amide bond. The disulfide may be formed between two molecules of the redox sensitive protein (leading to dimerization) or between two reactive thiolate of the same protein (leading to an intramolecular S–S bridge). In addition to these modifications, low degrees of oxidation may be stabilized by the formation of an intramolecular sulfenil–amide bond, in which the reactive thiolate reacts with the α-nitrogen of a close amino acid. Examples of each modification are reported.

sential roles in the recovery of oxidized PTP activity (38). Considering the differences in the p*K*a values of catalytic cysteines among PTPs (162), 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.

The regulation of PTK tyrosine phosphorylation through redox regulation of their associated PTPs is a well-recognized phenomenon (34). We can refer to this phenomenon as indirect PTK redox regulation through reversible PTP oxidation (Fig. 8). 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 signaling by avoiding its prompt inactivation by PTPs (Fig. 8). The functional significance of ROS-mediated PTP inhibition in GF signaling has been verified by blocking their accumulation. The first proof was provided by Sundaresan, who demonstrated that overexpression of catalase in vascular smooth muscle cells blocked PDGFR-induced tyrosine phosphorylation of ERK, as well as PDGF-induced DNA synthesis and migration (197). Second, interfering with H_2O_2 production through catalase loading of A431 cells considerably reduced the tyrosine phosphorylation level of EGFR (11). Catalase pretreatment eliminates the insulin-stimulated production of ROS as well as the inhibition of PTP1B, and was coupled with reduced tyrosine phosphorylation of insulin receptor (129). Finally, block of ROS production upon PDGF administration leads to the reduction of PDGFR tyrosine phosphorylation. Of note, the kinetics of ROS production, PTP redox inhibition, and receptor phosphorylation display an outstanding alignment, suggesting a stringent temporal correlation among these events (35). Therefore, it is likely that the redox inhibition of PTPs plays a vital role in RTK signaling, and that the salvage (via re-reduction) of the PTP catalytic activity after oxidation is followed by a dephosphorylation of activated receptors, thus terminating their signal. In this light, at least two PTPs have been indicated as responsible for the indirect redox regulation of PDGFR, namely LMW-PTP (36) and SHP2 (137), while PTP1B and TC45 have been proposed for the same role for insulin receptor (136).

Beside PTP redox regulation linked to GF signaling, a specific role of oxidants in integrin receptors signaling has now emerged. Extracellular matrix (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 three key regulators of cytoskeleton dynamics: PDGFR (33), the focal adhesion kinase (FAK) (37), and a GTPase activating protein for the GTPase Rho (p190RhoGAP) (149). Hence, upon integrin engagement, this redox circuitry leads, as a final event, to a proper execution of cell adhesion and spreading onto fibronectin (Fig. 9).

Besides RTKs, cytosolic PTKs are indirectly regulated through reversible inhibition of PTPs (Fig. 8). Several cPTKs are characterized by this kind of redox regulation, including FAK, Src, and Lyn. Oxidative stress induces a decrease in the distribution of FAK in focal contacts, without alteration of the integrins in adherent trabecular meshwork (212). An increase in FAK tyrosine phosphorylation owing to oxidative stress, has been described in different cells in human glioblastoma cell line T98G (191), in human umbilical vein endothelial cells (81), in bovine pulmonary artery endothelial cells (212), and in mesangial cells (227). More recently, 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 oxidated and inhibited in response to integrin engagement and in strict concomitance with the peak of ROS induced by ECM contact. Inhibitors of 5-LOX, which selectively block integrin mediated ROS generation, impede LMW-PTP oxidation/inhibition and as a result leads to FAK

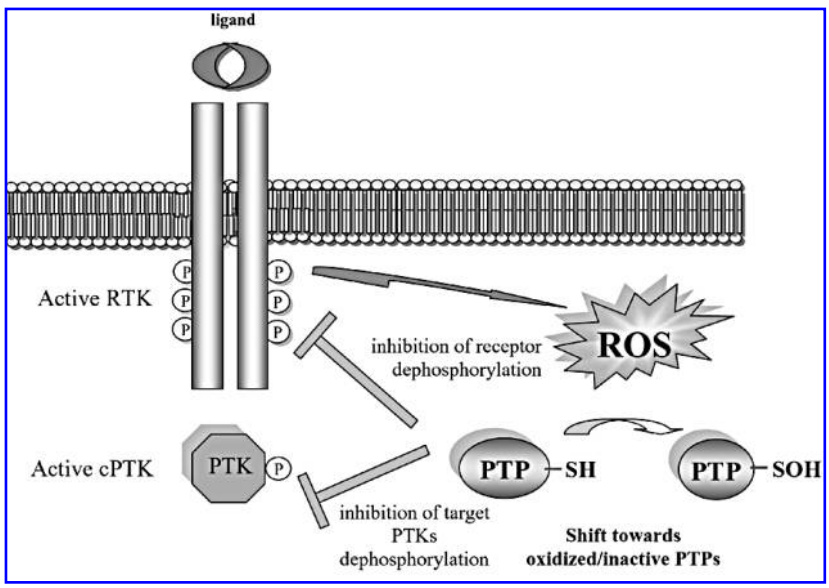

FIG. 8. RTK-induced production of ROS and their role as modulators of protein tyrosine phosphorylation through PTP redox regulation. Ligand-induced activation of RTKs leads to the production of ROS from NADPH oxidase. Among intracellular ROS targets are PTPs, which are catalytically inactivated by oxidation, hence allowing the sustained RTK/PTK phosphorylation/activation required for signaling propagation.

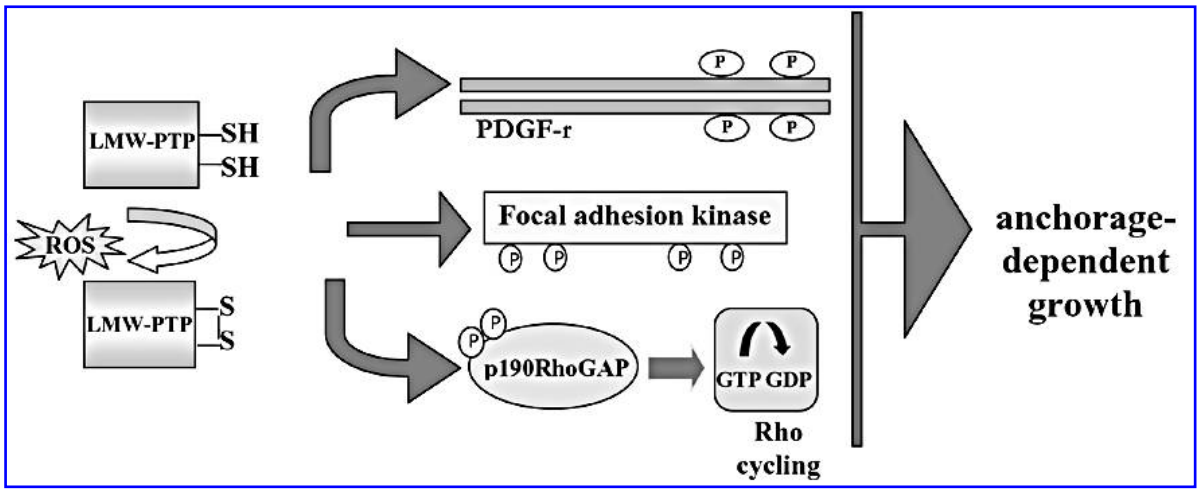

FIG. 9. The role of redox regulation of LMW-PTP in cytoskeleton assembly. Upon production of ROS by integrin signaling LMW-PTP becomes transiently oxidized/inactivated. This event leads to the transient hyperphosphorylation of three LMW-PTP substrates, namely PDGFR, FAK, and p190RhoGAP, leading in turn to Rho cycling. The transient phosphorylation of these proteins, all of which have been reported to exert a key role in cytoskeleton assembly and anchorage-dependent cell growth, is hence indirectly reliant on redox signaling.

downregulation. The redox regulation of FAK, through the inhibition of its dephosphorylation by LMW-PTP, leads to other key downstream events, including ERK phosphorylation, focal adhesion formation, and cell spreading, which are all significantly attenuated by inhibition of redox signaling (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 missing, both SHP2 (131) and PTEN (201) are excellent candidates for this function, due to their ability to dephosphorylate FAK.

Integrin regulation of Src kinase activity has a PTP-based component as well. In addition, 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 (the direct action of ROS on Src kinase will be described in the next paragraph). We find a redox-mediated downregulation of PTP activity during cell spreading onto ECM (unpublished data). This redox regulation of PTPs causes a hyperphosphorylation of Tyr416 in the activation loop of Src kinase. Further studies to identify this/these PTP/s are in progress. Although we cannot quantify the relative contribution to Src redox regulation of direct kinase oxidation or of PTP inhibition, we hypothesize that these two phenomena are concurrent in the achievement of the full activation of the Src kinase during cell/ECM contact.

In keeping with our observation on Src kinase, recently Singh *et al*. (188) reported that during B cell receptor signaling an other Src family kinase, Lyn, is redox regulated through inhibition of associated PTPs. These authors reported that SHP1 redox regulation cooperates with calcium signaling during the regulation of the extent of receptor downstream signaling, through PTP-mediated redox regulation of Lyn phosphorylation. They showed that the Ca2+ and reactive oxygen intermediates generated upon B cell receptor activation, are promptly engaged in a cooperative interaction, acting in a feedback way to strengthen the early signal generated. This cooperativity proceeds through the regulation of ROS production. Oxidants act by pulsed inactivation of receptor-coupled phosphatases, where the amplitude of this pulse is determined by oxidant concentration. In turn, the amount of PTP redox inhibition directs what fraction of receptor-associated Lyn kinases is activated. These outstanding data prove that the strength of the initial signal ultimately decides the final extent of extracellular-regulated signaling and the speed of its downstream diffusion.

Finally, the two main modifications that PTPs and PTKs undergo during signaling, oxidation and phosphorylation, may influence each other. In particular, a given PTP, whereas oxidized/inhibited, can be powerfully phosphorylated by a PTK, the oxidation being a security from autodephosphorylation. Conversely, as phosphorylation of several PTPs leads to an increase in their catalytic activity (123, 127, 172, 199), the re-reduction of a phosphorylated PTP may subsequently determines a superactivation of the PTP (Fig. 10). Hence, the first modification will be preparatory for the second one. The existing supporting data on this kind of dual regulation are very few, likely owing 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.* (137) 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. In agreement, the tyrosine phosphorylation of oxidized PTP1B, in response to IRK activation, was greatly improved (173). The redox-mediated inhibition of the autocatalytic activity of PTP1B was indicated as responsible of the hyperphosphorylation of the oxidized phosphatase. Recently, in our laboratory, Giannoni and co-workers evidenced that an exogenous oxidative stress, through oxidation/inactivation of LMW-PTP, enhances the tyrosine phosphorylation level of the enzyme, due to the inhibition of its auto-dephosphorylating activity. In particular, this increase in

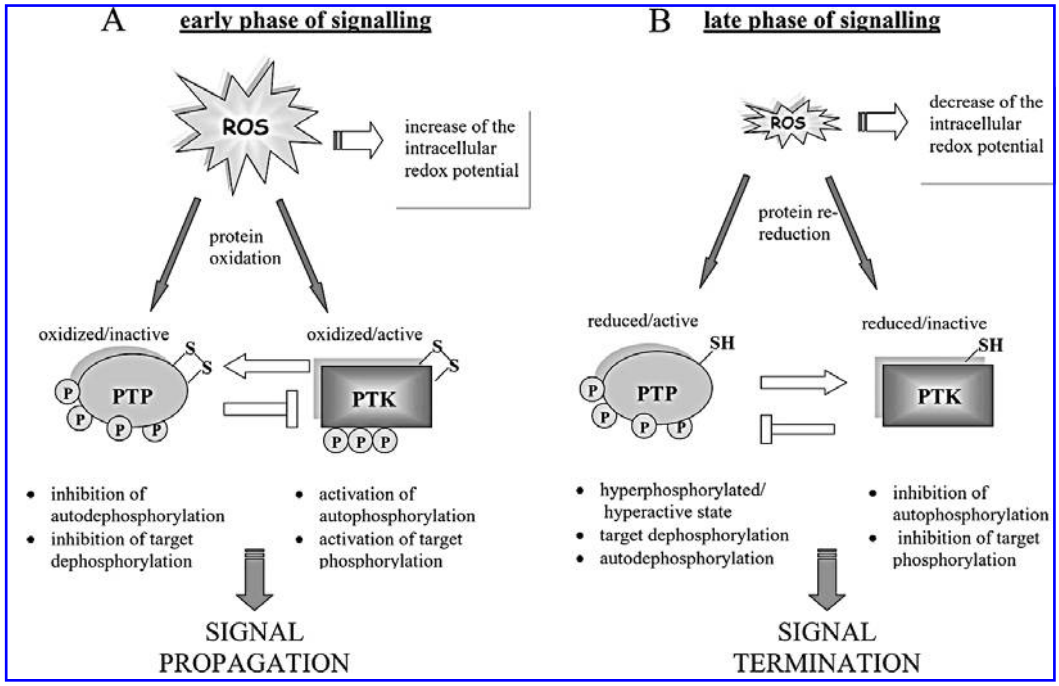

FIG. 10. Model of redox regulation of protein phosphorylation during signaling. During the early phase of redox-based signaling (*i.e.*, integrin and GF receptors activation), ROS production is able to oxidize both PTPs and PTKs, leading to opposing enzymatic activity regulation (*i.e.*, inhibition for PTPs and activation for PTKs). Oxidation of PTPs may lead to either the formation of an intramolecular S–S bridge or of a sulfenyl–amide bond (here we indicate a disulfide). 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 (here an intramolecular disulfide is indicated). (**A**) *Early signaling phase*: oxidized PTPs are concomitantly phosphorylated by PTKs, although this modification is for the moment without consequences due to redox-mediated PTP inactivation. 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 proteins. The complete inactivation of PTPs catalytic activity and the superactivation of PTKs lead as a final event to the propagation of signal transduction. (**B**) *Late signaling phase*: the decrease of intracellular oxidants allows the recover of the reduced state of both 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.

phosphorylation selectively involves Tyr132, which acts as a docking site for the adaptor protein Grb2. The redox-dependent enhancement of Grb2 recruitment to LMW-PTP ultimately leads to an improvement of ERK activation, likely eliciting a prosurvival signal against the oxidant environment (80).

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 superactive and guaranteeing an efficient termination of the signal (Fig. 10).

B. Redox upregulation of PTKs through oxidation and increase in tyrosine phosphorylation

Oxidants can also modulate the activity of protein kinases and, among these, PTKs. Once more in agreement with their opposing role to PTPs in the modulation of protein tyrosine phosphorylation, PTKs are activated by oxidation. However, the activation of 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. 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 (indirect PTK redox regulation, see above).

Oxidation-dependent activation of PTKs may affect membrane-bound kinases, as RTKs, or intracellular kinases, as Src tyrosine kinases, FAK, together with several ser-thr or dual specificity kinases, as MAPK or Akt and apoptosis signal-regulating kinase (170). A direct oxidation of RTK cysteines has been reported for IRK, EGFR, PDGFR, and Ret kinase. Schmid and co-workers reported that the best insulin sensitivity requires a process of 'redox priming' of the β-subunit of IRK, likely due to oxidation of IR β-chain sulfhydryl groups (179). This 'redox priming' of the IRK facilitates its autophosphorylation in the activation loop. In fact, 3D models of IRK revealed that the oxidation of any of the four cys-

teine residues 1056, 1138, 1234, and 1245 into sulfenic acid produces structural changes that bring Tyr1158 into close proximity to Asp1083 and make the catalytic site at Asp1132 and Tyr1162 more accessible (180). Hence, it is likely that cysteine oxidation enhances the tyrosine kinase activity of the IRK, thereby enhancing its downstream signaling.

The participation of cysteine oxidation in the activation of RTK is exemplified by cRet receptor, a cadherin-like domain containing RTK. 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 (174). Ultraviolet (UV)-mediated production of ROS of cells expressing c-Ret resulted in the dimerization of many c-Ret molecules on the cell membrane (107). Dimerization was mediated by the formation of a disulfide between the Cys residues of neighboring monomers, and the dimerized receptors were preferentially autophosphorylated. These results in c-Ret strong activation in response to ROS production during UV exposure. ROS contribute to the oncogenic potential of c-Ret as UV light irradiation induces superactivation of the constitutively activated Ret-MEN2A and Ret-MEN2B (107). Cys376 of c-Ret has been proposed as the key amino acid targeted by UV irradiation. Overexpression of superoxide dismutase prevented both the UV-mediated promotion of dimerization and the superactivation of Ret-MEN2A kinase, thereby suggesting that the UV-induced ROS induce intracellular domains of Ret to dimerize, causing kinase superactivation.

Finally, nitration of Tyr residues in PDGFR is observed in mild oxidant-treated cells, therefore signifying that ROS-induced modifications can occur to the receptor molecule and can be involved in the regulation of downstream pathways (67). In fact, this redox modification of PDGFR leads to Src-dependent activation of MAPK and Akt. 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 kinase activity of PDGFR-β, have been identified: Cys-822, positioned in the catalytic loop, and Cys-940, located in the C-terminal kinase subdomain. The role of these cysteines in tyrosine kinase activation of PDGFR is highly feasible as their individual substitution significantly reduced the activities of autophosphorylation and phosphorylation towards exogenous substrates of PDGFR (118).

Among intracellular PTKs, the Src tyrosine kinase, and some of the members of its family, are reported to be redox regulated. A first direct evidence of a redox-linked chemical modification of Src kinase has been obtained from *in vitro* experiments on nitric oxide (NO)-releasing agents (2). Exposure of Src to NO-releasing agents enhanced the catalytic activity of the kinase, either towards its autophosphorylation site or to its downstream substrates. NO-induced *in vitro* Src activation is independent from the phosphorylation on Src Tyr527, the C-ter autoinhibition site. In addition, a small portion of Src molecules are polymerized through S–S bond formation (2) The observation that the ROS targeted c-Ret cysteine residue is highly conserved in various nonreceptor PTKs, including Abl, Src, and Lck, suggests that it might also play a role in the activation of these enzymes. In keeping with this proposal, Src C-ter residues are crucial for either protein stability and cell transformation of this kinase (184). The relevance of *in vivo* Src redox regulation for anchorage-dependent growth is documented by our recent data (79). Src tyrosine kinase undergoes oxidation/activation, likely owing to an S–S bond between Cys245 and Cys487, respectively located in the SH2 and in the kinase domain of the Src molecule. We reported that the two C245A and C487A Src mutants behave as redox insensitive mutants, as they are unable to become activated during late adhesion onto ECM, while they show normal early activation (Fig. 11). Hence we proposed a model for Src activation in response to integrin engagement, involving a first redox independent phase, in which the kinase reaches a fair activation due to C-ter Tyr527 dephosphorylation likely mediated by RPTPα. Thereafter the kinase reaches a full activation state following oxidation of the two redox sensitive cysteines. Therefore, the tyrosine kinase c-Src is oxidized in response to cell attachment to ECM, and this modification leads to an enhancement of tyrosine kinase activity and activation of downstream Src-dependent signaling, likely due to the ROS burst owing to integrin engagement (79) (Fig. 12). In addition, our results suggest that ROS are key mediators of *in vitro* and *in vivo* v-Src tumorigenic properties. We reported

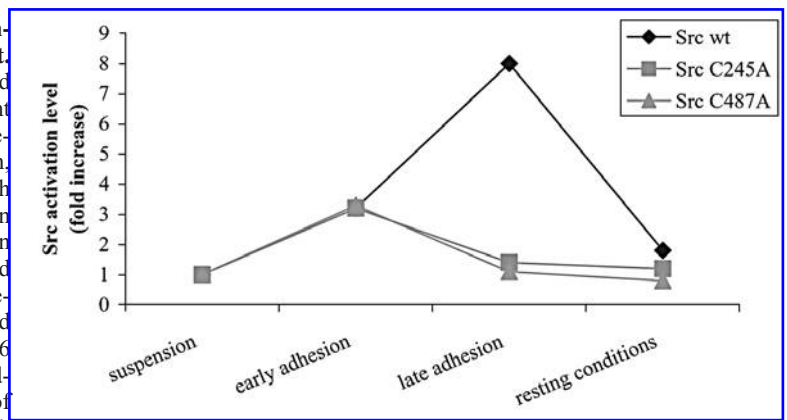

FIG. 11. c-Src activation during integrin-mediated cell spreading is redox dependent. NIH 3T3 cells were transfected with wt c-Src and with two cysteine to alanine oxidation-resistant mutants of the kinase (C245A and C487A, respectively) (79). Twenty four h after transfection, cells were serum starved for an additional 24 h before detaching and maintained in suspension for 30 min at 37°C. Then cells were either kept in suspension or seeded onto fibronectin-treated dishes for 10′ (early adhesion), 45′ (late adhesion), and 120′ (resting). Cells were lysed and Src activation was evaluated by anti-P-Tyr-416 Src immunoblot. Early integrin-mediated cell adhesion was concomitant with the formation of focal contacts, late adhesion was associated with the overall organization of actin stress fibres and the spreading of the adhering cells, while resting is associated with the conclusion of these events.

that both antioxidant treatments and the oxidant-insensitive C245A and C487A Src mutants greatly decrease invasivity, serum-independent and anchorage-independent growth of v-Src and Y527F-Src oncoproteins. As well, our data on tumor onset in athymic mice suggest that the *in vivo* oncoproperties on the two Src oncoproteins are redox dependent. Therefore, it is likely that the loss of C-terminal inhibition of v-Src and Y527F transforming Src gives rise to a still incomplete kinase activation, which is further enhanced by oxidation. Hence, in addition to the known phosphorylation/dephosphorylation circuitry, redox regulation of Src activity is required during both cell attachment to extracellular matrix and tumorigenesis.

C. Redox upregulation of PTPs through re-reduction and decrease of protein phosphorylation

Although the great majority of experimental evidences reported in literature concerns a negative regulation of PTPs due to regulated production of ROS in response to growth factor stimulation or integrin engagement, we recently observed an opposite regulation of a phosphatase in response to activation of an RTK (*i.e.*, EphA2 receptor kinase). Eph kinases are the largest family of RTK, and different biological functions have been attributed to them, including vascular development, branching morphogenesis, cell positioning, tissue border formation, cell migration, and axon guidance (61, 74, 113, 158, 224). They generally transduce repulsive, deadhesive, and antiproliferative signals and it is not surprising that many of the intracellular pathways that they transduce are opposite with respect to "positive" RTKs, including PDGFR and EGFR, generally leading to an attractive, adhesive, and proliferative response (16, 24, 53, 59, 66, 89, 91, 97, 139–142, 151, 158, 187, 200, 225). Among contrasting intracellular pathways exerted by "positive" and "negative" RTKs, we can mention MAPK and Rac1 activation. Indeed, proliferative RTKs cause a strong activation of both MAPKs and the

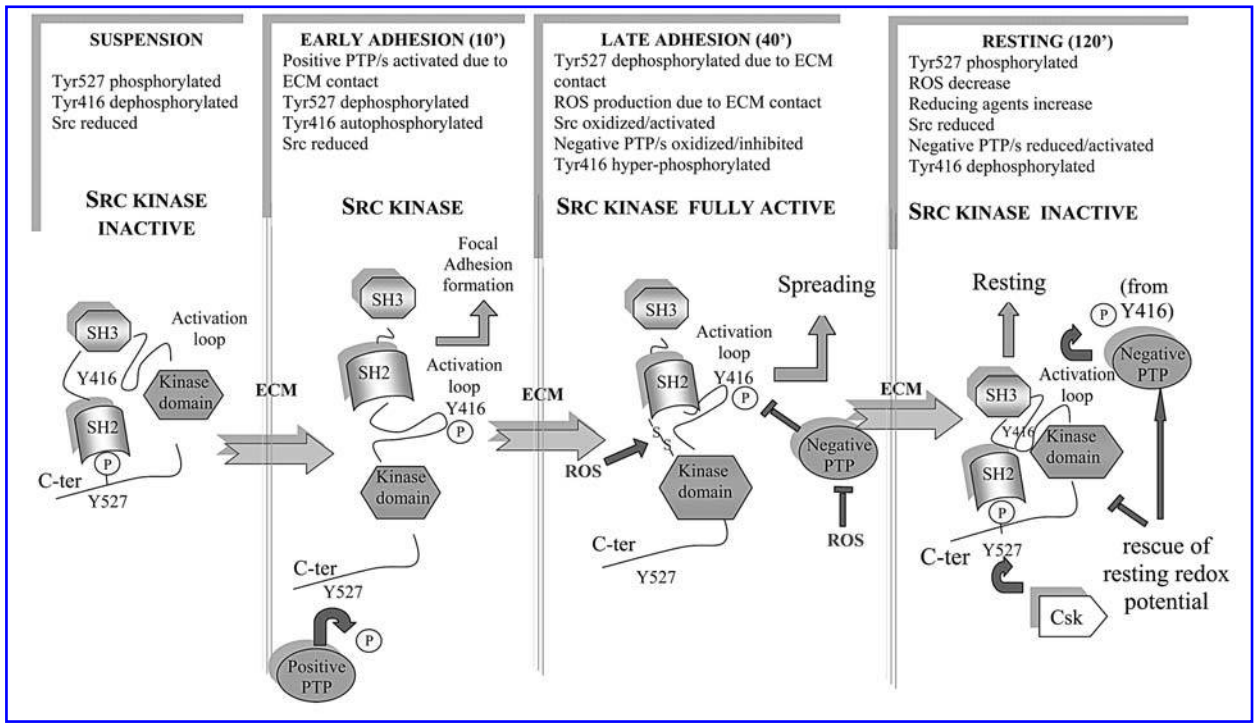

FIG. 12. Model of Src activation due to ECM contact. In suspended cells Src is in a "closed" inactive conformation, with the inhibitory Tyr527 phosphorylated and occupied with the SH2 domain. This blocked structure maintains the activation loop Tyr416 unavailable for the Src kinase domain and thus inhibiting autophosphorylation. At the early phase of integrin engagement activation of a positive PTP occurs (both R-PTPα and SHP2 have been proposed for such a role), leading to the dephosphorylation of Tyr527 and to the conversion of the kinase to an "open" conformation. Tyr416 is now accessible for autophosphorylation. Kinase activity increases with the following phosphorylation of several focal adhesion proteins and advancement of focal adhesion formation. At this stage of adhesion ROS concentration is not high enough to induce redox control of the Src kinase. At a later phase of cell adhesion, there is a strong increase in ROS production that leads to direct Src oxidation. Two cysteines within the Src kinase domain (C245) and the SH2 domain (C487) become oxidized and a disulfide bond is likely formed, thus leading the kinase and the SH2 domains in closer proximity. These events are associated with a strong increase of Src kinase activity. This phase of Src redox regulation is associated with cell spreading. In resting conditions, Tyr527 is newly phosphorylated by Csk kinase, inducing Src to return to a "closed" conformation. In addition, ROS decrease to a basal level, thus inducing both Src re-reduction and the rescue of previously redox-inhibited PTPs, concurring to dephosphorylation of Tyr416.

small GTPase Rac1, respectively guiding their proliferative and their motility responses. In contrast, several Ephs as EphA2 and EphB2, severely inhibit both MAPK and Rac1 activation. These events have been causally linked respectively to the antiproliferative and repulsive cues of ephrins.

Although the role of GF tyrosine kinase receptors in the generation of ROS has been largely accepted, Eph receptors have not been investigated in this feature. In keeping with their ability to inhibit Rac1, and with the key role of this small GTPase in the control of ROS production, we reported that EphA2 causes a decrease in ROS content upon stimulation with its ligand ephrinA1, owing to Rac1 inhibition (E. Giannoni *et al*., EMBO Workshop "Redox Signaling in human disease and aging." Roma 20–23 April 2006).

We recently reported that LMW-PTP, through dephosphorylation of EphA2 kinase, negatively regulates the repulsive response elicited by ephrinA1, thereby confirming the relevance of the net level of tyrosine phosphorylation of Eph receptors (157). The phosphatase interferes with the ephrin-mediated regulation of proliferation, motility outcome, cell adhesion, and the retraction of the cell body (157). Our data indicate that LMW-PTP undergoes a redox regulation during ephrin-mediated cell repulsion. In agreement with the strong decrease of intracellular ROS elicited by EphA2 ligation, LMW-PTP is reduced/activated during ephrin treatment (Fig. 13A). Interestingly, the variation of enzymatic activity of LMW-PTP follows the kinetics of ROS cellular content due to ephrinA1 administration. We observed that LMW-PTP redox upregulation is causally linked to EphA2 dephosphorylation, as indicated by the kinetic of receptor phosphorylation after ligation of ephrinA1 (Fig. 13A). We detect an extreme early activation of the kinase followed by a late EphA2 dephosphorylation, in strict concomitance with the redox upregulation of LMW-PTP. Therefore, during the repulsive signaling elicited by ephrinA1, the redox regulation that LMW-PTP undergoes is opposite with respect to that observed during growth factor or integrin receptor signal transduction (*i.e.*, a transient shift towards the reduced/active form). In our opinion, this feature is in agreement with the ability of ephrins to contrast both the proliferative GF-elicited signal and the adhesive integrin-elicited message (142, 233, 234).

By comparison of the kinetics of EphA2 tyrosine phosphorylation, of ROS cellular content, and of LMW-PTP enzymatic activity, we developed a model, shown in Fig. 13B. The kinetics of EphA2 tyrosine phosphorylation in response to its agonist may be separated into two parts: an early and a late signaling phase. In the early activation of signaling the maximum of the tyrosine phosphorylation level of EphA2 receptor takes place, owing to ligand-dependent increase in local receptor concentration and leading kinase activity to a threshold where transphosphorylation will overcome the counteracting PTP activity. At the same time, both ROS and LMW-PTP retain basal levels, likely due to concomitant redox signaling from cell adhesion. During late ephrin signaling, a Rac1-dependent message causes the ROS decline and the consequent LMW-PTP redox activation gives rise to a dephosphorylation of the EphA2 kinase, thus terminating the signal. Once again, repulsive Eph receptors behave inversely with respect to proliferative GF-receptors: while "positive" GF-receptors and adhesive integrin receptors cause a positive feedback loop based on PTP redox downregulation, the "negative" Eph repulsive receptors set up a negative feedback loop based on PTP redox upregulation.

D. Redox-dependent ligand-independent activation of RTKs

Besides their function as downstream modulators of RTK signaling, ROS act as upstream key molecules in RTK *trans*-activation, leading to a ligand-independent signal transduction. RTK ligand-independent *trans*-activation has been reported to be redox dependent in three features: (a) during exogenous oxidant delivery by lymphocytes, macrophages, or upon treatment with ROS producing agents as alkylating molecules, UV, and heavy metals; (b) during the integration of cross-talking signals elicited by G-protein coupled receptors (GPCRs) or integrins and several RTKs; and (c) during lateral propagation of EGF transduced signal.

A large body of experimental evidence suggests that, in addition to endogenous ROS development, extracellular oxidants affect RTK signaling. Indeed, radiations, exposure to metals, alkylating agents, and environmental oxidants have been reported to activate RTKs in a ligand-independent manner (218). Again, oxidation and following inactivation of PTPs have been indicated as responsible for RTK *trans*-activation by extracellular oxidants. Cysteine modification in the PTP catalytic site has been proposed for both UV- and alkylating agents-induced RTK *trans*-activation (84, 109), confirming a key role for PTP redox regulation/inhibition in the control of RTK tyrosine phosphorylation, even in the absence of the natural ligand. Furthermore, in both phagocytes and lymphocytes, the NADPH oxidase complex generates oxidants involved in host defense and inflammation. Hence, the activation of this response may result in pro-oxidant conditions for bystander cells (10). In addition to lymphocyte and macrophage activation, several cardiovascular diseases are characterized by a state of excess oxidative stress associated with enhanced production of ROS within the arterial wall (100). It is an intriguing possibility that these pro-oxidant backgrounds cause a redox regulation of RTKs (through PTP inhibition) in neighboring cells during inflammation. However, there is yet no direct evidence for such a mechanism. If held true, this interpretation might shed a new light on cell signaling during the inflammatory reaction or cardiovascular diseases with important pathophysiological and therapeutic implications.

Second, stimulation of other membrane receptors, as GPCRs or integrins can lead to RTK *trans*-activation (Fig. 14). The GPCR agonists lysophosphatidic acid, angiotensin-II, carbachol, thrombin, endothelin, serotonin, bradykinin, and isoproterenol induce the tyrosine phosphorylation and consequent activation of several RTKs such as PDGFR, EGFR, and insulin-like growth factor receptor (50, 51, 63, 82, 133). The involvement of ROS has been proposed as a causal event for intracellular GPCR-mediated RTK *trans*-activation (52, 122, 170). Interestingly, activation of different GPCRs leads to the generation of H_2O_2, suggesting that consequent PTP redox inactivation, as well as PTK redox activa-

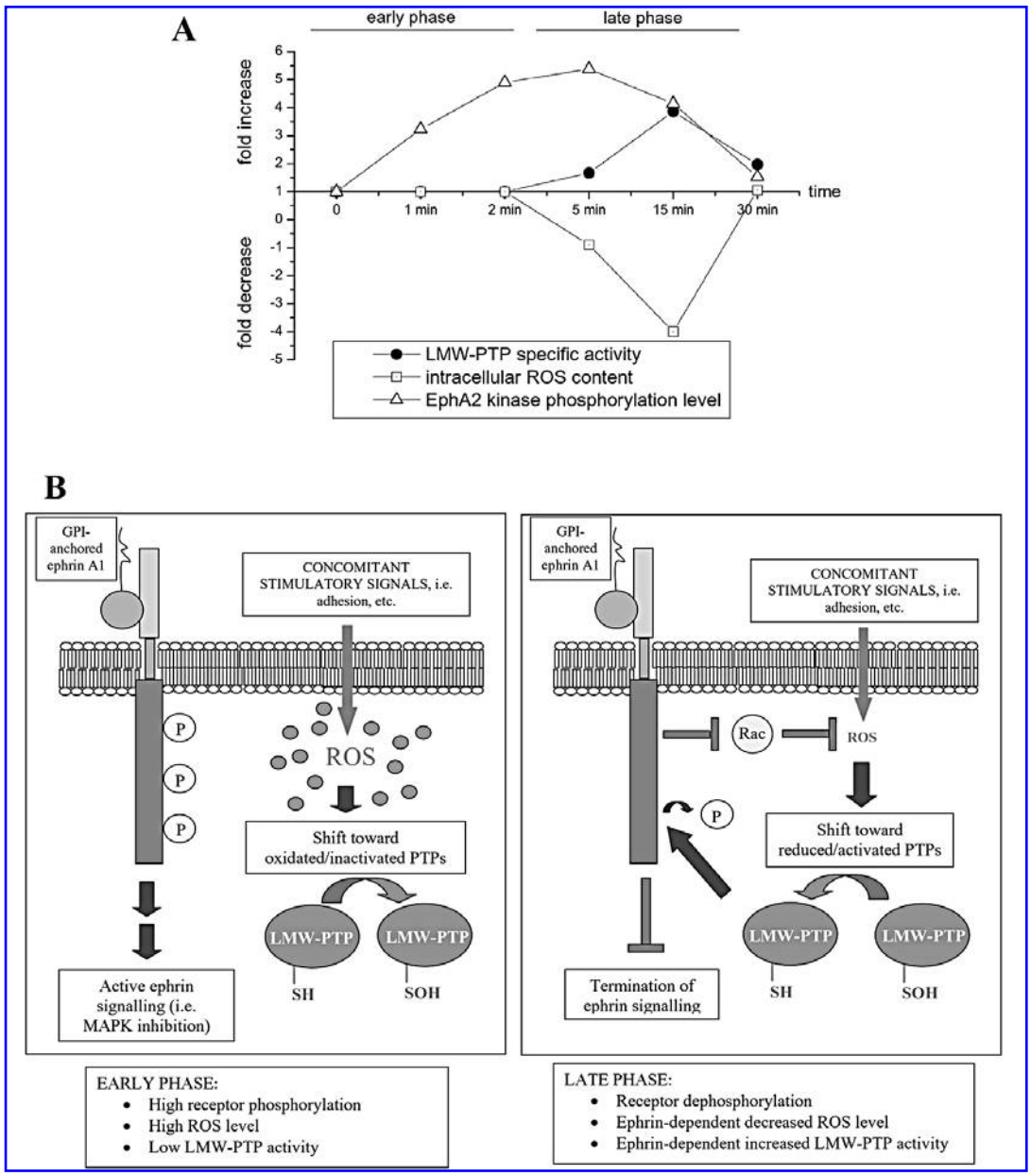

FIG. 13. (A) Kinetics of ephrin-mediated redox circuitry events. ROS content (156), EphA2 receptor tyrosine phosphorylation level (157) and LMW-PTP enzymatic activity (37) have been evaluated during ephrinA1 stimulation. During the early phase of Eph RTK signaling (1′–5′ from ligand stimulation), we observe a great increase in EphA2 tyrosine phosphorylation, while both ROS content and LMW-PTP activity were unaffected. Thereafter, during the late phase (5′–30′ from ligand administration), we reported a strong decrease of ROS content, concomitant with an increase of LMW-PTP enzymatic activity and a strong decrease of EphA2 receptor phosphorylation. The increase/decrease with respect to unstimulated samples is indicated. (**B**) Proposed model of ephrin-mediated redox circuitry during repulsive signal. The activation of EphA2 receptor in response to its agonist may be separated into two parts, that is an early (*left*) and a late (*right*) signaling phase. In the early activation of signaling, the maximum of the tyrosine phosphorylation level of EphA2 receptor takes place, owing to ligand-dependent increase of kinase activity, thus reaching a threshold where *trans*-phosphorylation of EphA2 overcomes the counteracting resting LMW-PTP activity. At the same time both ROS and LMW-PTP retain basal levels, due to the resting ratio between oxidized LMW-PTP (likely due to concomitant redox signaling from cell adhesion) and reduced LMW-PTP. This high EphA2 phosphorylation level guarantees that ephA1 signaling (*i.e.*, MAPK inhibition), takes place. During late ephrin signaling, a Rac1-dependent ephrin-mediated signal causes the ROS decline and the consequent redox shift towards LMW-PTP reduction\activation. Now LMW-PTP activity prevails over the EphA2 kinase activity, thus leading to dephosphorylation of the EphA2 receptor and terminating the signal.

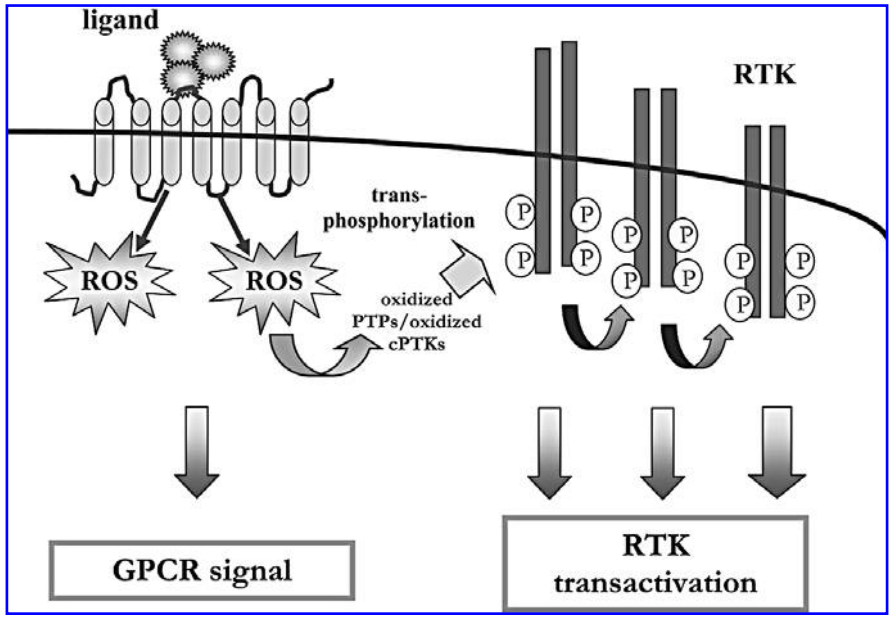

FIG. 14. Model for redox-mediated RTK *trans*-activation by GPCRs. Many GPCR have been reported to elicit a ROS burst during signaling. The subsequent redox-mediated inactivation of PTPs associated with bystander RTKs can cause their activation, by means of shifting the phosphorylation equilibrium towards RTK tyrosine phosphorylation.

tion, can contribute to intracellular GPCR-induced *trans*-activation of RTKs. Actually, the prevention of H_2O_2 accumulation by antioxidants blocked RTK stimulation and downstream ERK activation, in cells treated with several GPCR ligands, as lysophosphatidic acid (LPA), angiotensin II, or serotonin, thereby suggesting a role for redox PTP inhibition in the intracellular RTK *trans*-activation pathways starting from these GPCRs (52, 82, 208). For instance, angiotensin II has been proposed as responsible for the *trans*-activation of both PDGF and EGF receptors, respectively, in smooth muscle cells and epithelial cells (202, 208, 214). In addition, an intriguing study by Heeneman and co-workers (87) demonstrates that angiotensin II *trans*-activation of PDGFR involves an unique pathway that regulates a population of tyrosine kinase receptors different from those stimulated by endogenous tyrosine kinase ligand. The mechanisms that underlie such *trans*-activation remain unclear, although both redox activation of Src and Pyk2 kinases, and PTP redox inhibition, have been suggested to play a role (65, 71, 110, 111, 128, 176, 189, 208).

Besides GPCR-cross talk, RTKs can be activated *in trans* by other neighboring RTKs during their ligand-dependent signaling. For instance, cross-communication between the Met receptor tyrosine kinase and EGFR has been proposed to involve redox signaling (73).

Adhesion molecules are able to associate with RTKs and trigger their ligand-independent activation (43). This complex interaction is believed to be a key step of protection from *anoikis* of anchorage-dependent cells (68, 104, 143, 145, 164, 181, 190, 198). The kinase Src has been indicated as responsible for the cross-talk between EGF and integrins, likely causing the ligand-independent integrin-dependent EGFR *trans*-phosphorylation (144). We propose that redox signaling plays a key role in the interplay between integrin-activated Src and the ligand independent EGFR *trans*-phosphorylation (Fig. 15A). In keeping with this hypothesis, we observed that the protection from *anoikis* exerted by integrin-mediated adhesion of nontransformed cells, is redox dependent (Fig. 15B) as indicated by its sensitivity to antioxidant and lipoxygenase inhibitors. In addition, protection from *anoikis* of these cells is likely correlated with redox regulation of Src kinase, as indicated by the inability of the two redox-insensitive Src mutants to sustain *anoikis* defense (Fig. 15C). Therefore, the cross-talk between EGF and integrin receptors is again redox dependent, even if in this interplay the redox-sensitive protein is likely not a PTP, but a PTK.

Finally, the last feature in which hydrogen peroxide has been implicated as a key molecule leading to ligand-independent RTK activation, is lateral propagation of EGF signaling waves (169). Reynolds and colleagues demonstrated that in response to locally administered EGF, lateral propagation of EGFR phosphorylation occurs at the plasma membrane, signifying an early amplification step in EGFR signaling. They show evidence that this phenomenon is caused by a redox inhibition of PTPs coupled with RTK activity, leading to ligand-independent *trans*-activation of neighboring EGFR molecules (Fig. 16). These authors propose that in EGF ligand-dependent signaling, there is a threshold response leading to the maintenance of phosphorylated receptors in plasma membrane regions that are not exposed to ligand. Nevertheless, before generalizing this phenomenon to the signal propagation of all RTKs, these outstanding data need to be extended to other ROS-producing RTKs.

III. CONCLUDING REMARKS

The emerging picture underlines that opposite redox regulation of PTPs and PTKs is a key point during vital processes such as cell proliferation, migration, and adhesion.

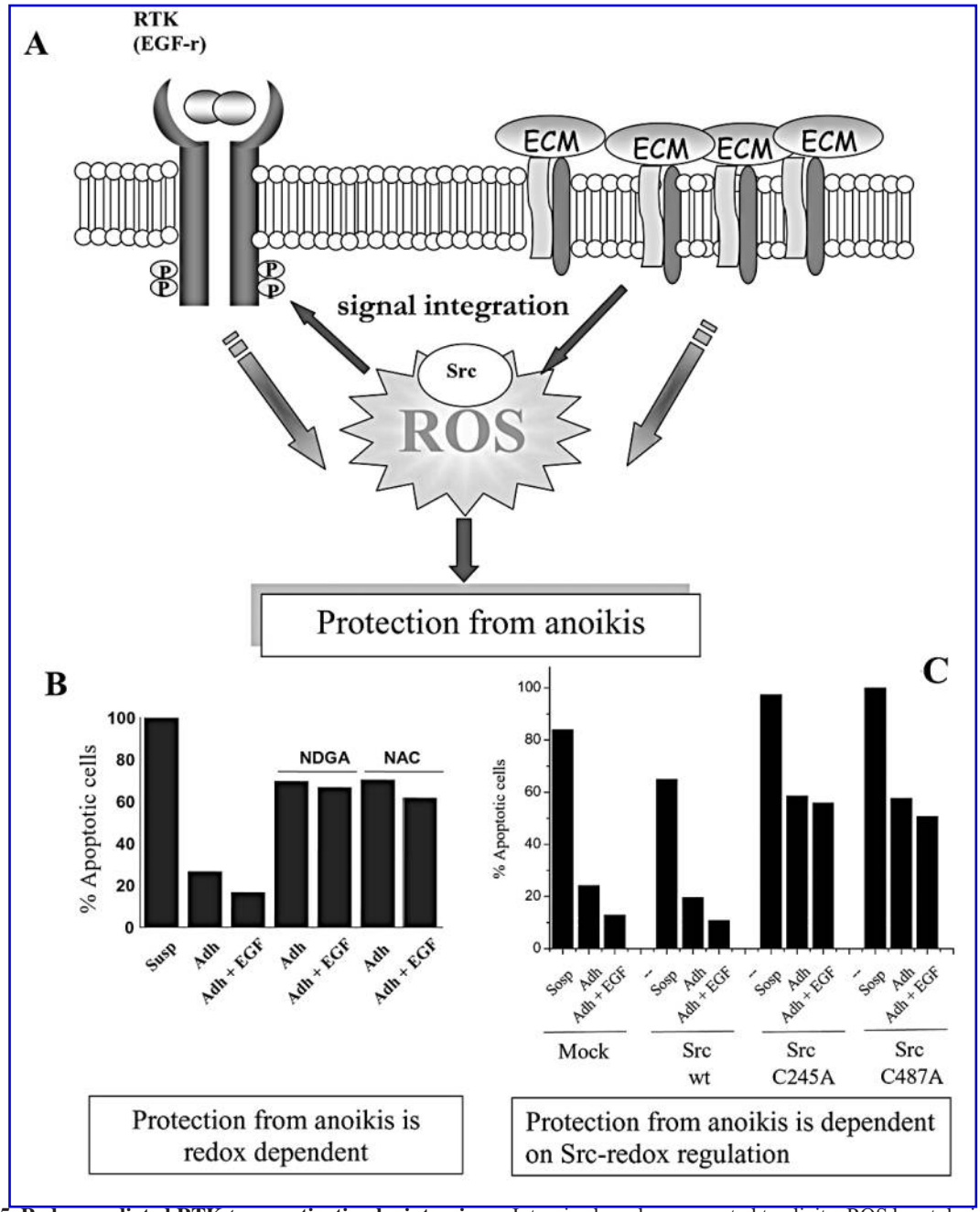

FIG. 15. Redox-mediated RTK *trans*-activation by integrins. Integrins have been reported to elicit a ROS burst during signaling and to cause RTK *trans*-activation. (**A**) The mechanism, at least for EGFR, has been reported to be Src-dependent and we speculated that Src redox regulation may have a key role for the associated protection from *anoikis*, depending on the integration of signals from RTKs and ECM. (**B**) Role of redox signaling in *anoikis* protection exerted by ECM proteins. ECV304 endothelial human cells were detached with EDTA 2.5 m*M*, maintained in suspension for 20 min and then seeded onto fibronectin-coated dishes, in presence of 100 ng/ml EGF, with or without pretreatment with 10 μ*M* NDGA or 20 m*M* NAC. After 24 h the percentage of cells undergoing apoptosis has been evaluated by flow cytometry. (**C**) Role of Src redox regulation in *anoikis* protection exerted by ECM proteins. NIH 3T3 cells were transfected with wt c-Src and with two cysteine to alanine oxidation-resistant mutants of the kinase (C245A and C487A, respectively) (79). Twenty four h after transfection, cells were serum starved for an additional 24 h before being treated as in B.

We can outline a complex redox circuitry whereby, upon ROS increase due to cell adhesion and/or growth factor stimulation, oxidative inhibition of PTPs, together with direct oxidation of some PTKs leading to their activation, supports the activation of several downstream outcomes. By this way, the antagonistic balance between PTPs and PTKs, respectively being inhibited and activated by ROS, become actually indistinct as both these modifications cooperate in achieving a properly executed cell response to extracellular stimuli.

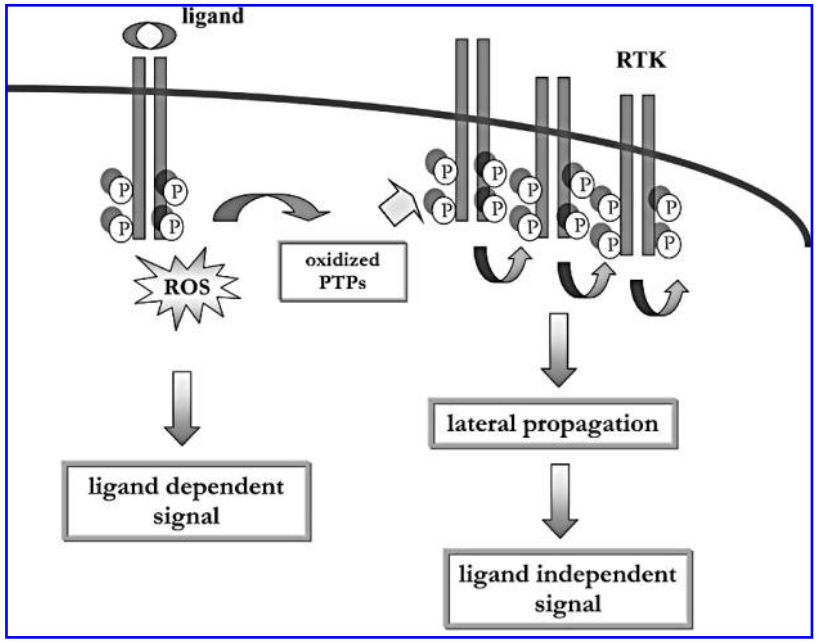

FIG. 16. Model for RTK later signal propagation. This model has been reported for EGFR, although it may virtually be extended to any other RTK, whose phosphorylation has been indicated as redox-dependent. In this model the ROS produced by the subset of ligand-stimulated RTK molecules cause the redox-mediated inactivation of PTPs associated with bystander RTKs. This event causes their ligand-independent activation, by means of shifting the phosphorylation equilibrium towards RTK tyrosine phosphorylation. Hence, the propagation of a signaling wave originating from ligand/RTK interaction sites occurs, thus leading to the maintenance of phosphorylated receptors in plasma membrane regions that are not exposed to ligand.

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Date of first submission to ARS Central, July 25, 2006; date of final revised submission, September 1, 2006; date of acceptance, September 2, 2006.

This article has been cited by:

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