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Comprehensive Invited Review

Protein Tyrosine Phosphorylation and Protein Tyrosine Nitration in Redox Signaling

HUGO P. MONTEIRO,[1] ROBERTO J. ARAI,[1] and LUIZ R. TRAVASSOS[2]

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ABSTRACT

Reversible phosphorylation of protein tyrosine residues by polypeptide growth factor–receptor protein tyrosine kinases is implicated in the control of fundamental cellular processes including the cell cycle, cell adhesion, and cell survival, as well as cell proliferation and differentiation. During the last decade, it has become apparent that receptor protein tyrosine kinases and the signaling pathways they activate belong to a large signaling network. Such a network can be regulated by various extracellular cues, which include cell adhesion,

[1]Department of Biochemistry/Molecular Biology and CINTERGEN; and [2]Experimental Oncology Unit (UNONEX), Department of Microbiology, Immunology and Parasitology, Universidade Federal de São Paulo, São Paulo, Brazil.

agonists of G protein–coupled receptors, and oxidants. It is well documented that signaling initiated by receptor protein tyrosine kinases is directly dependent on the intracellular production of oxidants, including reactive oxygen and nitrogen species. Accumulated evidence indicates that the intracellular redox environment plays a major role in the mechanisms underlying the actions of growth factors. Oxidation of cysteine thiols and nitration of tyrosine residues on signaling proteins are described as posttranslational modifications that regulate, positively or negatively, protein tyrosine phosphorylation (PTP). Early observations described the inhibition of PTP activities by oxidants, resulting in increased levels of proteins phosphorylated on tyrosine. Therefore, a redox circuitry involving the increasing production of intracellular oxidants associated with growth-factor stimulation/cell adhesion, oxidative reversible inhibition of protein tyrosine phosphatases, and the activation of protein tyrosine kinases can be delineated. *Antioxid. Redox Signal.* 10, 843–889.

I. INTRODUCTION

In the environment in which we live, humans constantly balance the use of oxygen as an energy source and as a cellular injury source. Reactive oxygen species signal cascades that determine cell growth, cell death, mitogenesis, angiogenesis, and carcinogenesis. Reactive oxygen species are generated as byproducts of oxidative phosphorylation in the mitochondria and as products of the phagocytic NADPH-oxidase (NOX2) activation, and also of xenobiotic metabolism. The oxidative stress can lead to DNA mutations, protein damage, and lipid peroxidation (145).

Defense and sensing mechanisms are thus essential for the cell to cope with alterations in the normal intracellular redox status. Antioxidant enzymes superoxide dismutase (SOD), catalase, and glutathione (GSH) peroxidase may protect cells against the molecular species that are collectively termed as reactive oxygen species (ROS), which include superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), and singlet oxygen ($^1O_2$). Thiol-containing GSH, thioredoxins, glutaredoxins, and peroxiredoxins also play an essential role in keeping redox homeostasis and regulation (145).

Cellular responses that are initiated and controlled by ROS or by molecular oxygen (O_2) and by reactive nitrogen species (RNS; *e.g.*, nitric oxide—·NO and peroxynitrite—$ONOO^-$) are within the research area called redox signaling. Such signaling has been described in eukaryotic cells and bacteria.

How is redox signaling translated into a cellular response that may ultimately represent cell viability or cell death? Transcription factors can be regulated by O_2 tension. The classic example is that of transcription factor hypoxia-inducible factor 1 (HIF-1). The expression of genes targeted by HIF-1 is triggered at very low O_2 tension. These genes are related to cell proliferation and viability, erythropoiesis and Fe metabolism, as well as vascular development and remodeling (332). Another redox-based regulation of transcription depends on the interactions of transcription factors such as AP-1, NF-κBl, and Elk1 with thioredoxin (Trx). Nuclear translocation of Trx was identified as a general response of cells under oxidative and nitrosative stresses (3, 11, 163, 164, 240, 372). Modification of transcription factors by ROS also is possible. With the transcription factor OxyR from *Escherichia coli*, the reduced form of the protein binds to DNA but cannot induce transcription. Posttranslational modifications involving the participation of ·NO and H_2O_2 resulting in the formation of S-NO, S-OH and S-glutathionyl adducts are actively involved in signaling events. (160, 198).

Hydrogen peroxide is important for transduction of signals by platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin, and TNF-α (262, 349), and it functions by oxidizing cysteine residues, depending on the amino acid sequence and acidic pK_a. The protein tyrosine phosphatases (PTPs) signature active-site motif, HCXXGXXRS/T, has a Cys with low pK_a (4.7–5.4) that is ionized at neutral pH. The resulting thiolate anion contributes to the formation of a thiol-phosphate intermediate in the catalytic mechanism of PTPs. The reversible oxidation to sulfenic acid of this Cys by H_2O_2 added extracellularly or generated intracellularly by stimulation with epidermal growth factor inactivates PTPs, resulting in phosphorylation and proliferation (88, 226).

In addition, the reducing protein Trx is redox regulated. S-nitrosation of Cys69 on Trx was described under basal conditions in endothelial cells. This redox-based posttranslational modification of Trx is required for scavenging ROS and for preserving the redox regulatory activity of Trx (143). Furthermore, tyrosine nitration, a covalent posttranslational protein modification derived from the reaction of proteins with $ONOO^-$ and other nitrating agents, appears to be a selective process relevant to redox signaling (266).

Transmission of information to the cells through redox signaling is therefore a very complex procedure involving a network of receptors and catalytic steps that have been recognized, and the corresponding molecules and their active forms have been identified.

The present review focuses on tyrosine phosphorylation and tyrosine nitration, as key processes of redox signaling. Oxidative modifications such as Cys S-nitrosation and Tyr nitration on small G proteins, kinases, and phosphatases are studied as sensors and ways to modulate/mediate tyrosine phosphorylation–dependent cellular signals.

II. RECEPTOR-LIKE AND CYTOPLASMIC PROTEIN TYROSINE KINASES

Cells efficiently respond to external signals by promoting the reversible phosphorylation of intracellular proteins. Target proteins are phosphorylated by protein kinases and have their phosphate groups removed by protein phosphatases. Among protein kinases, the most abundant are the ones that transfer a γ-phosphate group from the ATP molecule to the amino acids Ser, Thr, and Tyr. Although phosphotyrosine levels represent a small proportion of the total cellular content of phosphoamino acids ($<<1\%$), they play a major role in cell signaling.

In addition, members of the phosphatidylinositol 3 kinase (PI3K) family also exhibit protein-Ser/Thr kinase activity, and some kinases possess dual specificity for both Tyr and Ser/Thr

(37). Regarding protein tyrosine kinases (PTKs), they are encoded by ~100 genes; many of them are related to receptor-like PTK (RPTK), whereas others encode nonreceptor, cytoplasmic PTK (308) (Fig. 1).

RPTKs are distributed in 20 families, and 19 of them encompass receptors that are single monomeric polypeptide chains in the absence of their specific ligand. Prototypic receptors for some of these families are the epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), and the fibroblast growth factor receptor (FGFR). Constitutive dimers of two polypeptide chains linked to each other by disulfide bonds is the structural characteristic of a single family of receptors represented by the insulin and the insulin-like growth factor (IGFI) type I receptors (37).

RPTKs display a large variety of structures and functions, and they are organized in three major domains:

1. The extracellular domain, presenting a variable structure that recognizes different ligands;
2. A hydrophobic single α-helix transmembrane domain that spans the membrane; and
3. A cytoplasmic domain featuring catalytic and regulatory domains. Catalytic domains of RPTKs are relatively conserved structures, with homology ranging from 32% to 95% (170).

Hormones, growth factors, and cytokines bind to their specific RPTKs, which undergo oligomerization. As an additional consequence, ligand binding to an RPTK promotes the intermolecular phosphorylation in trans of one receptor and its neighbor, within the activation segment of the kinase domain (see Fig. 1). It is well documented that most RPTKs display 1–3 Tyr residues in the activation loop (A-loop) of the catalytic domain (147). Autophosphorylation of the Tyr residues located in the A-loop results in conformational changes, which will facilitate the access for ATP and protein substrates (261). The stimulated kinase domain phosphorylates other tyrosine residues outside the catalytic region of the receptor, usually in the regulatory region, providing docking sites for cytoplasmic signaling proteins (326).

Among cytoplasmic PTKs, we focus on the Src family of these kinases (1, 74, 311) and on the family of FAK, the focal adhesion kinases (69, 75). We also comment on a third family of cytoplasmic PTKs, the Syk/ZAP-70 family, which is ex-

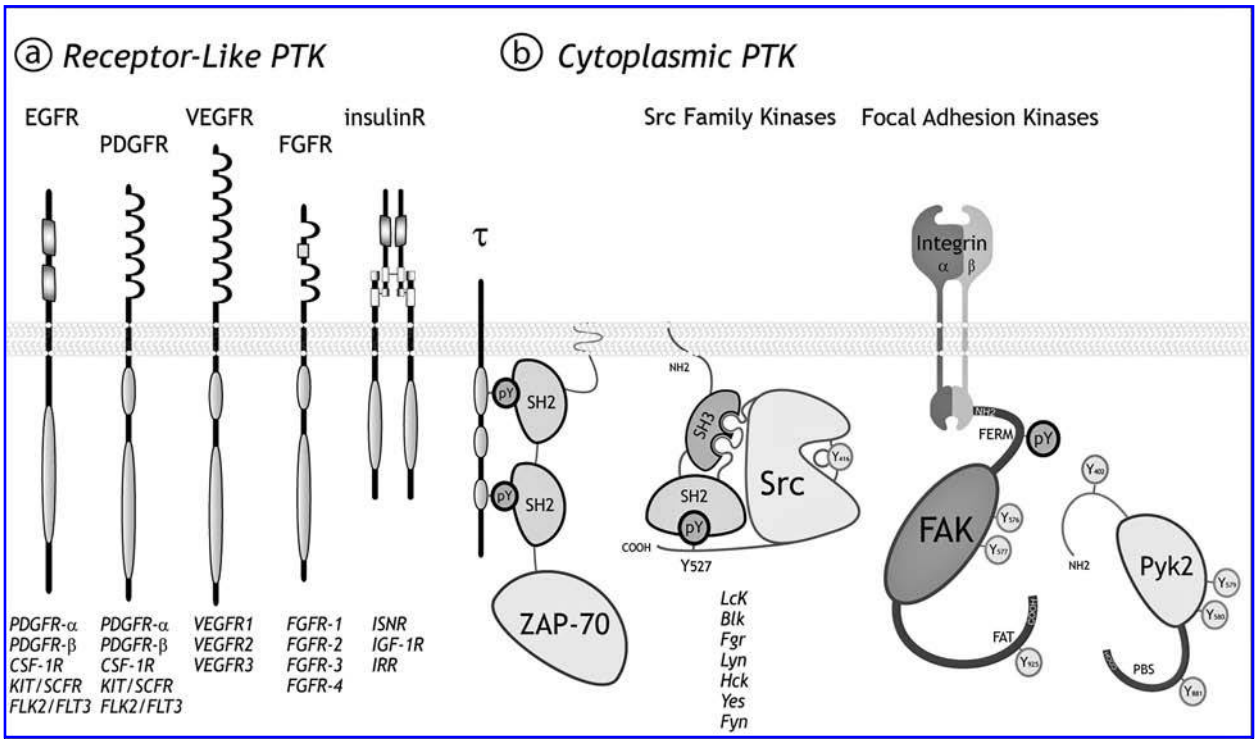

FIG. 1. Protein tyrosyne phosphorylation is an essential component in intracellular signaling. Protein tyrosine kinases (PTKs) are receptor-like PTKs (RPTKs) and nonreceptor cytoplasmic PTKs. RPTKs have an extracellular ligand binding domain, a hydrophobic single transmembrane domain, and a cytoplasmic domain with catalytic and regulatory sites. The cytoplasmic PTK ZAP-70 is only expressed in T lymphocytes and natural killer cells. It associates with the T-cell receptor (τ) through its SH2 domains. Besides ZAP-70, this family of PTKs also is represented by Syk which is not shown in the figure. The Src family of kinases comprises 11 members. Eight members are represented in the figure. Blk, Fgr, Hck, Lck, and Lyn are expressed in haematopoietic cells. Src, Yes, and Fyn are expressed in all cell types. FAK and proline-rich tyrosine kinase 2 (Pyk2) are cytoplasmic focal adhesion kinases. FERM domain links FAK to integrin receptors. EGFR, epidermal growth factor receptor; PDGFR, platelet derived growth factor receptor; VEGFR, vascular endothelial growth factor receptor; FGFR, fibroblast growth factor receptor; insulinR, insulin receptor and insulin growth factor 1-receptors.

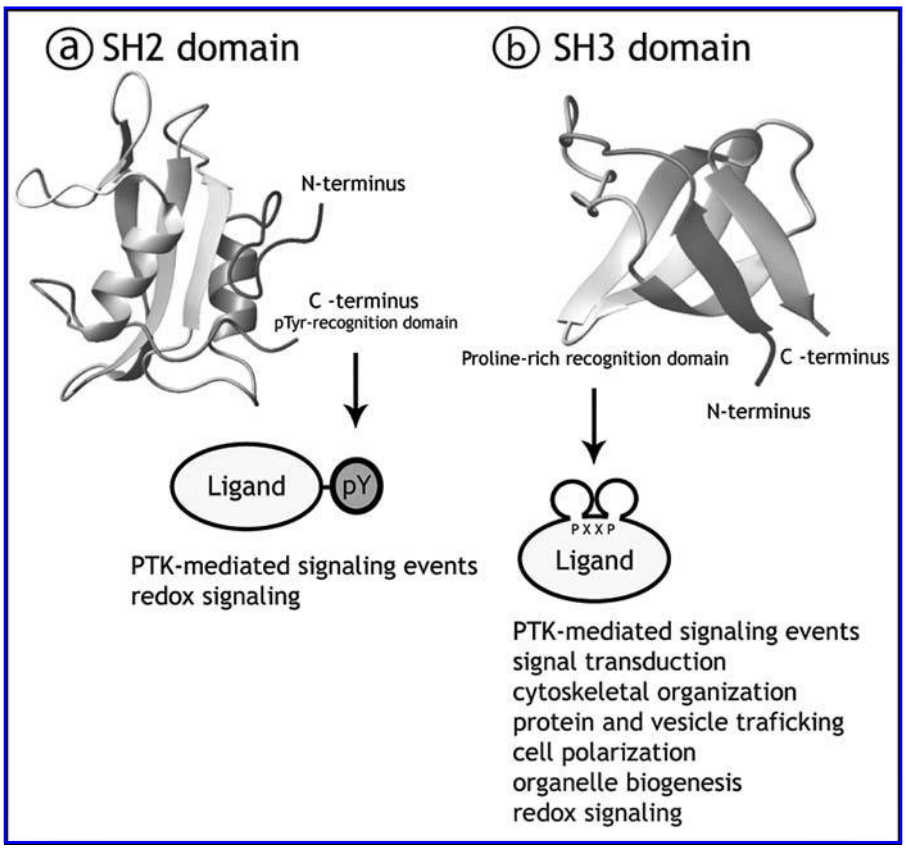

FIG. 2. Ligands of SH2 and SH3 domains and PTK signaling events. Src kinase homologous SH2 and SH3 domains primarily recognize pY residues and proline-rich sequences, respectively, regulating PTK-mediated signaling events, redox signaling, and other biological activities.

pressed in lymphocytes and other hematopoietic cells (221, 12) (see Fig. 1).

The Src family of kinases in humans comprises 11 members highly related to Src. LcK, Blk, Fgr, Lyn, and Hck are expressed preferentially in hematopoietic cells. Brk is expressed in colon, prostate, and small intestine. Frk occurs in bladder, brain, breast, colon, and lymphoid cells. Srm is found in keratinocytes. The other three members, Src, Yes, and Fyn, are more generally expressed (311). The Src family of PTK participates in a great number of signaling pathways, including cell activation and mitogenesis (35). Rearrangements on the cytoskeleton and oxidative stress conditions modulate Src kinase activity (1, 93). Overexpression of Src stimulates tyrosine phosphorylation of focal adhesion proteins, leading to loss of cell adhesion and morphologic changes associated with cell transformation (53). FAK and proline-rich tyrosine kinase 2 (Pyk2) are the two components of this family of cytoplasmic PTKs (37). FAK, a major component of the focal adhesion complexes, becomes phosphorylated on specific tyrosine residues on Src overexpression and activation (320). A central catalytic domain flanked by large amino and carboxy-terminal domains essentially constitutes FAK. The amino-terminal domain mediates the interactions of FAK with membrane-associated proteins. This domain, also known as the FERM domain, plays a major role in the interactions of FAK with integrin receptors, and with growth factor receptors such as the receptors for PDGF and EGF. Indirect interactions with integrins and direct interactions with growth-factor receptors characterize the association of FAK with both receptors (338, 339).

Conversely, the carboxyl/terminal domain encompasses three praline-rich regions and the focal adhesion target (FAT) domain (148). The carboxy-terminal FAT domain contains binding sites for paxillin and talin, which will form together with vinculin, actin, FAK, Src, and the docking protein p130CAS, the focal adhesion complex (127).

Pyk2, the second member of the family, is highly expressed in cells from the central nervous system and in hematopoietic cells. Stimulation of monocytes with the differentiation factor M-CSF/CSF-1 promotes tyrosine phosphorylation of Pyk2 and cell differentiation (149). FAK and Pyk2 are structurally related, exhibiting 48% amino acid identity (16). Pyk2 is rapidly phosphorylated on tyrosine on the increase of the intracellular levels of calcium and the activation of PKC (230).

The Syk/ZAP-70 family of cytoplasmic PTKs includes two identified members: Syk and ZAP-70 (221,12). Syk is highly expressed in B lymphocytes but is also found in lower expression levels in other hematopoietic cells including T cells. ZAP-70 is expressed only in T lymphocytes and natural killer cells (190).

In general, cytoplasmic PTKs, like their receptor counterparts, display a catalytic domain and modular structures that interact with other signaling proteins and lipids. These modular structures are extremely versatile in their binding properties. They are able to engage several ligands, providing the means to assemble signaling complexes as well as acting as regulators of individual components of the formed complex (290).

Phosphorylated tyrosine residues are specifically recognized by modular structures of 100 amino acids, known as SH2 domains. Named after a homologous domain found in Src kinase, such domains recognize phosphotyrosine residues in the ligand by peptide sequences of three to six amino acids within the carboxy-terminal region (288). SH2 domains serve specific func-

tions; they require phosphotyrosine sites in their ligands and are dedicated to PTK-mediated signaling events (291) (Fig. 2).

The Src-homologous type 3 (SH3) domains are sequences ranging from 50 to 75 amino acids and primarily bind to proline-rich sequences (327). In comparison with SH2 domains that are dedicated only to tyrosine kinase signaling, SH3 domains participate in a greater number of biological activities. These domains regulate processes such as signal transduction, cytoskeletal organization, protein and vesicle trafficking, cell polarization, and organelle biogenesis (343, 250) (see Fig. 2).

SH2 and SH3 domains serve as prototypes for a large and growing family of modular protein domains found in all intracellular signaling proteins. For instance, phosphorylated tyrosines may also be recognized by the so-called PTB or protein tyrosine–binding domains. Differently from SH2 domains, PTB domains recognize both phosphorylated and nonphosphorylated Asn-Pro-X-Tyr motifs. In addition, PTB domains have a structural fold similar to domains that bind to proline-rich motifs (EVH1 domains) and to domains that bind phospholipids such as the pleckstrin homology (PH) domains (290). However, increasing experimental evidence points to the participation of SH2 and SH3 domains in redox signaling. Therefore, we focus our attention on both domains in our discussion on tyrosine phosphorylation and tyrosine nitration in redox signaling. For additional information on domains and signaling, readers are encouraged to refer to recent excellent reviews (289, 292, 299).

III. SH2 AND SH3 DOMAINS AND THE ASSEMBLY OF SIGNALING CASCADES

A. Overview of cytoplasmic protein tyrosine kinase signaling pathways

RPTKs and the cytoplasmic PTKs Src and FAK, with phosphorylated Tyr residues, function as docking sites for proteins containing SH2 and SH3 domains. In the case of Src kinase and the other members of the Src family of PTK, besides phosphorylation, myristylation, and palmitoylation, are posttranslational modifications, essential for anchoring these kinases to the inner leaflet of cell membranes. In Src, the membrane targeting signal is followed by SH3 and SH2 domains, by the kinase domain, and by a carboxy-terminal region that contains a conserved regulatory tyrosine phosphorylation site (Fig. 3). Therefore, the phosphophorylation status of Src and interactions between specific tyrosine residues and SH2 domains are important regulators of Src activity. Two major phosphorylation sites present on Src were described as regulators of kinase activity: Tyr416 is a positive regulatory autophosphorylation site, and Tyr527 is a negative and critical regulatory carboxy-terminal phosphorylation site (246) (see Fig. 3). Strong evidence has been obtained for the interaction of phosphorylated Tyr527 residue with the SH2 domain of Src. This interaction results in the suppression of the tyrosine kinase activity of Src (1, 49). Various PTKs and protein tyrosine phosphatases (PTPs; discussed in later sections of this review article) have been implicated in the control of the phosphorylation status of Tyr527. Among the PTKs, the carboxy-terminal Src kinase (Csk) was firstly identified by Okada and Nakagawa (280). Csk is ubiquitously expressed, contains SH2 and SH3 domains, is constitutively active, and does not require phosphorylation for activity (281). Adapter proteins control the enzyme activity tethering it to the plasma membrane through contacts between a phosphotyrosine residue in the adapter protein and the SH2 domain of Csk (192). Csk can then phosphorylate and downregulate Src and other members of the Src family of PTK.

In addition, the interaction between the SH2 domain with the carboxy-terminal pTyr527 and the binding of the SH3 domain to a Pro-X-X-Pro motif in the kinase domain also suppresses Src kinase activity (35). Stimulation of Src kinase activity involves the "opening" of the structure on dephosphorylation of Tyr527 and the release of SH2 and SH3 domains that can bind to tyrosine phosphorylated proteins and proteins containing a Pro-X-X-Pro motif, respectively. Receptor-like PTPs such as RPTPα displaces p-Tyr527 from the Src SH2 domain, mediates its dephosphorylation, and leads to the activation of Src kinase. This regulatory mechanism is common to all members of the Src family PTKs. Indeed, p-Tyr505 in Lck, a member of the Src family of kinases expressed in hematopoietic cells, is displaced from its SH2 domain by the tyrosine phosphatase CD45, resulting in kinase activation (351).

Regarding the positive regulatory phosphorylation site, Tyr416, it is located within the A-loop. Phosphorylation of Tyr416 provokes its displacement from the substrate-binding pocket, allowing the kinase access to substrates (246) (see Fig. 3).

Conformational changes associated with the activation of Src kinase follow the association of Src with FAK. Moreover, the activation of FAK requires autophosphorylation at Tyr397, which occurs after integrin engagement and concomitant activation of Src. Phosphorylated Tyr397 is a high-affinity binding site for Src, resulting in the recruitment and binding of Src kinase to FAK (321). Src, then, phosphorylates FAK on residues Tyr576 and Tyr577 within the FAK catalytic domain, promoting full kinase activity of the enzyme. The phosphorylated tyrosine residues on FAK serve as docking sites for SH2 domain containing proteins. Besides Src, it is well documented that phosphorylated Tyr397 appears to be the main binding site also for PI3K, phospholipase C-γ (PLC-γ?), and the adapter proteins Shc and Grb7 (287).

Src can further phosphorylate FAK on Tyr925 at the carboxyl-terminal domain. Phosphorylated Tyr925 now is a docking site for the adapter protein Grb2, connecting both kinases to the Ras/Raf/MEK/ERK MAP kinase signaling pathway (252). It was also demonstrated that Src could phosphorylate Tyr861 on FAK, an important event for cell migration (235). Experiments with cells expressing dominant negative mutants of FAK, FAK-null cells, and cells overexpressing a PTP, PTEN, which dephosphorylates FAK, established an essential role for FAK on integrin-mediated cell migration (131, 141, 285).

In addition to cell migration, FAK is essential for integrin signaling. In contrast to growth-factor receptors that display intrinsic tyrosine kinase activity, integrins, which are the major receptors in the extracellular matrix, are not involved with these activities. Oligomerization of integrins with formation of clusters leads to activation and autophosphorylation of FAK. Src, FAK, and structural proteins associated with phosphorylated FAK, such as paxillin, vinculin, talin, and actin, will constitute the focal adhesion complex (69) (Fig. 4).

Recently, the Schlaepfer group (339) provided experimental evidence for the ability of FAK to integrate growth-factor re-

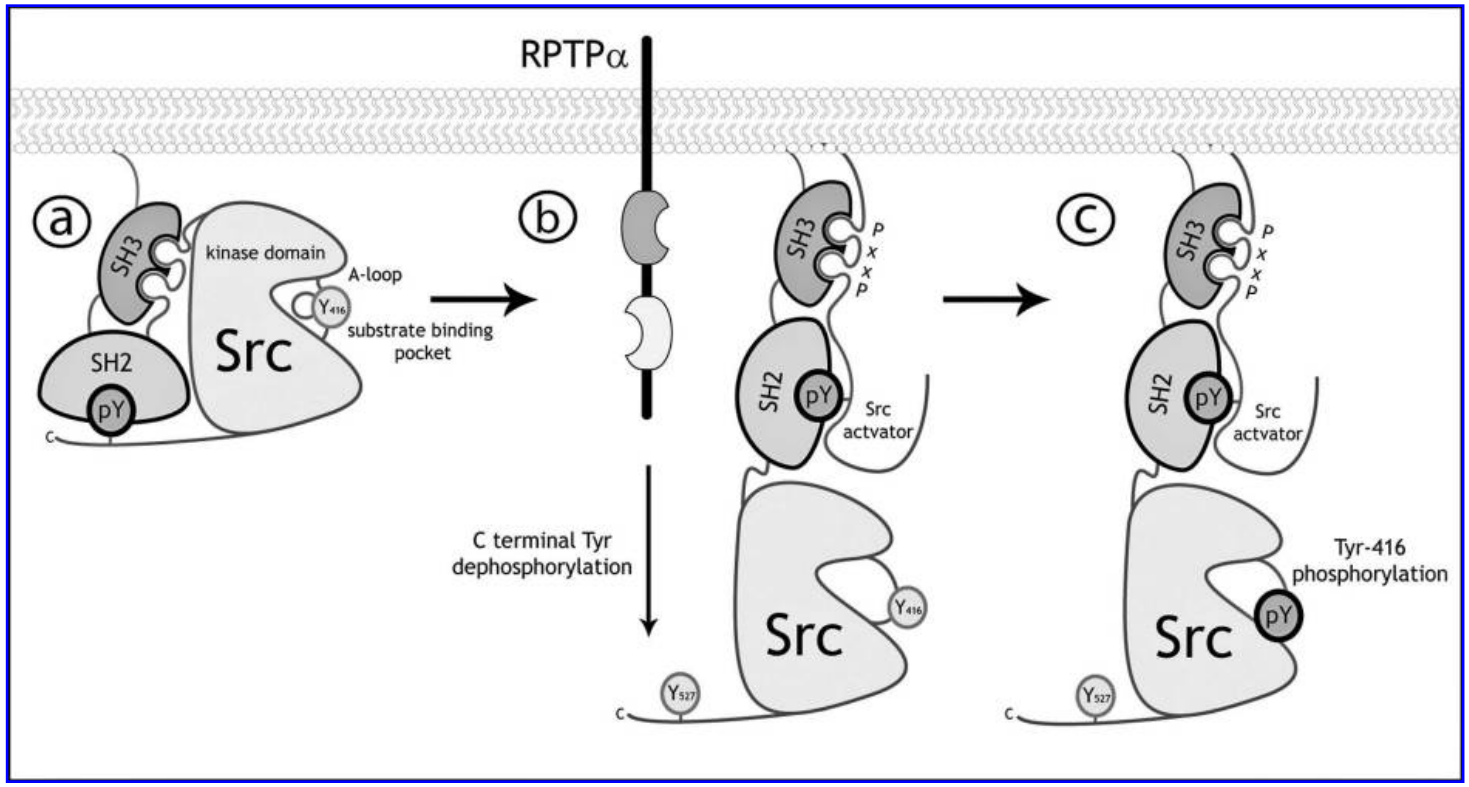

FIG. 3. Transition from inactive to active Src kinase upon tail dephosphorylation, stimulation by binding proteins, and Tyr416 phosphorylation. Src kinases are normally inactive by autoinhibitory interaction between the SH2 domain and carboxy-terminal Tyr527 residue distal from the catalytic domain. Dephosphorylation of Tyr527 releases SH2 and SH3 attached by a flexible linker and they become free to interact with cellular proteins or activators. Phosphorylation of Tyr416 at the A-loop displaces it from the substrate binding pocket allowing kinase access to the substrate.

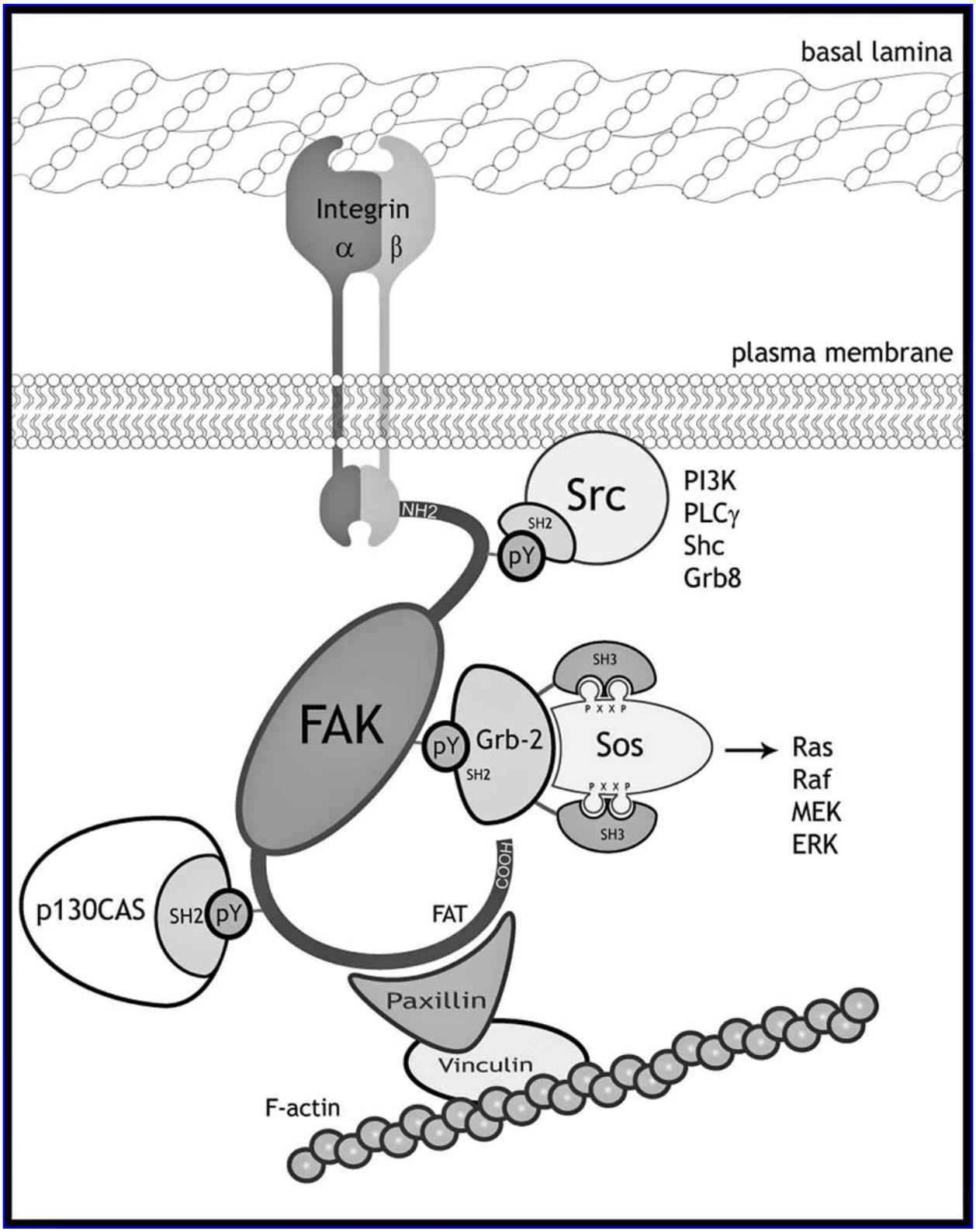

FIG. 4. The focal adhesion complex. Clusters of integrins lead to activation and autophosphorylation of FAK, Src, FAK and structural proteins paxillin, vinculin, talin, and actin constitute the focal adhesion complex. Src phosphorylated FAK is a docking site for the adapter protein Grb2 connecting the two kinases to the Ras/Raf/MEK/ERK MAP kinase signaling pathway.

ceptor–mediated signals with signals coming from integrin-mediated adhesion processes. In addition, it was demonstrated that FAK integrates signals from Src and the Src family of PTKs coordinating adhesion dynamics with survival signaling in cancer cells (252).

Membrane immunoglobulins and T cell–receptor signaling pathways are mediated by the cytoplasmic PTKs Syk and ZAP-70, respectively (12, 221). The two SH2 domains in the Syk/ZAP-70 PTKs interact to pTyr residues within immunoreceptor tyrosine-based activation motifs of antigen-receptor complex proteins. On such interactions, the PTKs become active (189) . In addition, activation of ZAP-70 requires Lck (Src family PTK)-mediated phosphorylation of one or more tyrosine residues (165).

B. Signaling downstream from receptor protein tyrosine kinases

Ligand-induced activation and phosphorylation of RPTKs such as the EGFR and PDGFR make use of SH2 and SH3 domains to form linear signaling pathways. Specifically, for the SH2 domains, the sequence context of the autophosphorylated site determines the nature of the SH2-containing protein that will engage the autophosphorylated receptor. Therefore, specific residues in the SH2 domains of PLC-γ, Src, and Grb2 ultimately determine which protein will be recruited by a phosphorylated RPTK (290).

Three basic mechanisms underlie the signal-transduction events that develop from the interactions between the activated RPTK and the SH2/SH3 containing signaling proteins. The first involves the engagement of the SH2 domains on PLC-γ with phosphotyrosine residues on the cytoplasmic domain of the EGFR and PDGFR (196, 245). After engagement with the receptor, PLC-γ is phosphorylated on tyrosine and activated. The activated enzyme catalyzes the production of two second messengers, diacylglycerol and inositol triphosphate, essential for PKC activation (277).

The second mechanism involves the activation of Src kinase by the PDGFR. Once activated, the PDGFR dimerizes and undergoes autophosphorylation on several tyrosine residues. Besides other signaling proteins, Src, Fyn, and Yes are recruited to these tyrosine phosphorylated residues (105). The activated PDGFR phosphorylates Src at Tyr138 within its SH3 domain (51) and Tyr213 located within its SH2 domain (350). Phosphorylation on both sites may disrupt the intramolecular interactions that stabilize the inactive conformation of Src, resulting in kinase activation. Activated Src will phosphorylate several proteins, including Stat3 and the adapter protein Shc. These phosphorylated proteins allow Myc transcription and DNA synthesis (74, 370).

A third manner of transmitting signals from activated RPTKs involves the participation of adapter proteins such as Grb2. Grb2 interacts with a Pro-X-X-Pro motif on Sos, a guanine nucleotide–exchanging factor (GEF) for p21Ras, through its SH3 domain. The newly formed complex, Grb2/Sos, engages a specific phosphotyrosine residue on the cytoplasmic domain of the RPTK and translocates to the plasma membrane (Fig. 5). The complex Grb2/Sos associates with the adapter protein SHC phosphorylated on tyrosine through its SH2 domain. The multiprotein complex now functions as a GEF for p21Ras converting the inactive GDP-associated GTPase into its active GTP-associated conformation (177, 341).

Besides cell proliferation, RPTK activation could also lead to survival signaling pathways mediated by the Ser/Thr kinase Akt, which plays a major role in cell survival and tumorigenesis (364). At the structural level, Akt features an amino-terminal PH domain, a central kinase domain with an activation-loop Thr308 phosphorylation site, and a conserved, regulatory serine phosphorylation site, Ser473, near the carboxy-terminal region. The most accepted model for recruitment and activation of Akt involves the activation of an RPTK with recruitment and activation of PI3K and formation of inositol triphosphates. These phospholipids recruit Akt to the plasma membrane through interactions with its amino-terminal PH domain. At the plasma membrane, Akt is phosphorylated and activated by the Ser/Thr protein kinase PDK-1 (see Fig. 5). Recent publications list 13 substrates for active Akt, among them the forkhead transcription factor, the proapoptotic Bcl2-family member Bad, and the cyclic-AMP response element–binding protein as well-known participants of cell-survival signaling pathways (83).

The concept of linear-signaling pathways is the simplest way of describing a signaling cascade initiated by an RPTK. In recent years, it has become clear that RPTK-initiated signaling pathways are part of a signaling network that can be regulated by multiple extracellular cues, such as cell adhesion, agonists of G protein–coupled receptors, Ser/Thr receptors for cytokines, and stress signals (322). Adhesion of cells to a surface triggers concomitant signaling events involving co-clustering of integrins with RPTKs. The integrated signal is conveyed by FAK, which has its tyrosine phosphorylation levels stimulated simultaneously by RPTKs and integrins mediated by Src kinase. FAK interacts through its amino-terminal domain with EGFR- or PDGFR-activated complexes with integrins, placing this cytoplasmic PTK as a receptor-proximal component of both integrin and RPTK signaling pathways (339) (see Fig. 5). Thrombin, endothelin, lysophosphatidic acid, and angiotensin have in common the fact that although all of them are agonists of several G protein–coupled receptors, they stimulate tyrosine phosphorylation of EGFR and PDGFR (54). Coupling of EGFR and receptors of the transforming growth factor β (TGF-β) has also been documented (248). TGF-β receptors are a group of cytokine receptors that have Ser/Thr activity and, on activation, form a hetero-tetrameric complex composed of receptors type I and II. Stimulation of TGF-β receptors results in Ser/Thr phosphorylation and activation of SMAD proteins. These proteins are transcriptional regulators that, on activation, translocate to the nucleus and promote the transcription of target genes (248). It was shown that coupling of EGFR and TGF-β receptors negatively regulates TGF-β signaling. EGF induces tyrosine phosphorylation of SMAD proteins at specific sites, leading to inhibition of nuclear translocation and transcriptional activity (85, 210).

RPTKs are activated by stress conditions such a hyperosmolarity and membrane depolarization. Oxidative stress conditions promoted by ultraviolet radiation, redox active transition metals, increasing levels of ROS and RNS also stimulate RPTKs activities (262). As we discuss in the later sections of this review article, accumulated experimental evidence gives support to a direct relation between tyrosine phosphorylation–mediated signaling pathways and the redox environment.

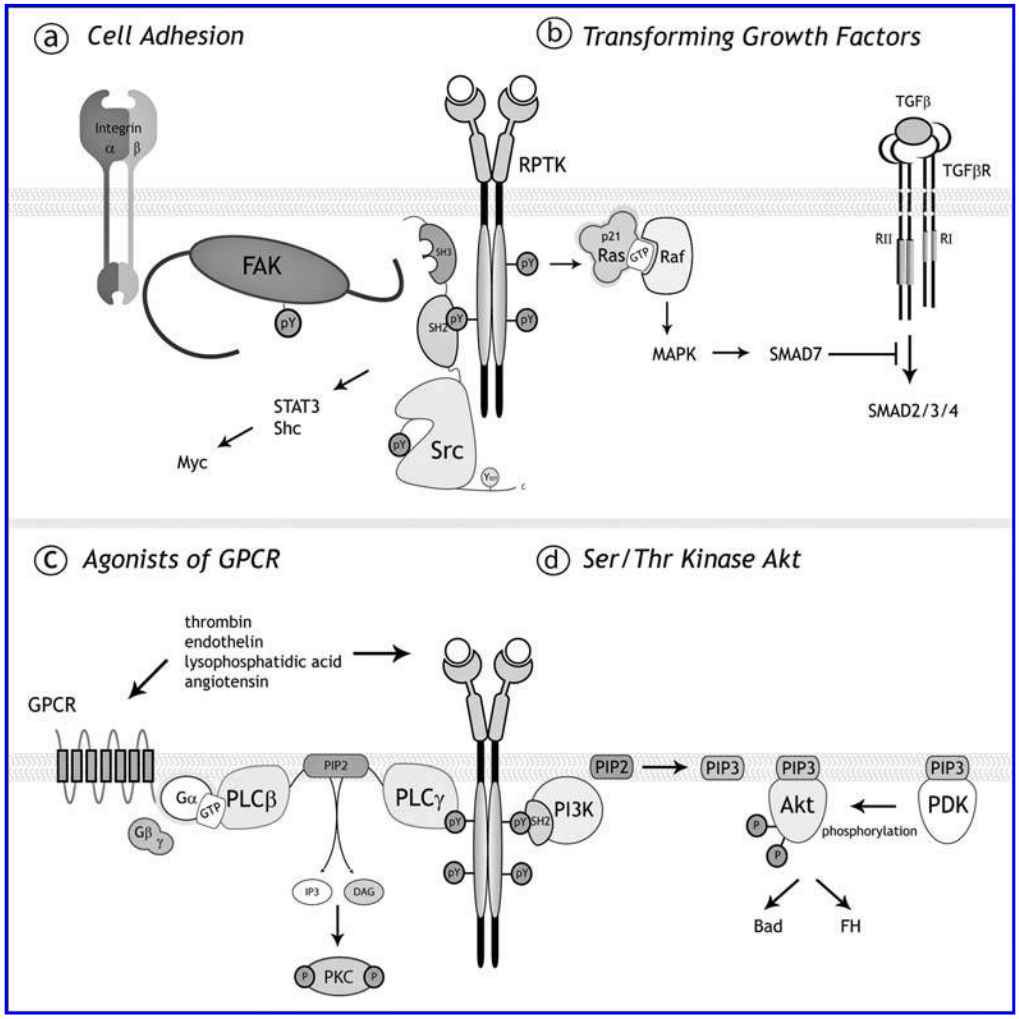

FIG. 5. RPTK-initiated signaling pathways can be regulated by cell adhesion, agonists of G-protein-coupled receptors, and Ser/Thr receptors for cytokines. Adhesion of cells to a surface triggers co-clustering of integrins with RPTKs. The integrated signal is conveyed by FAK, which has its tyrosine phosphorylation stimulated by RPTKs mediated by Src kinase. Thrombin, endothelin, lysophosphatidic acid, and angiotensin are agonists of several G-protein-coupled receptors and stimulate tyrosine phosphorylation of EGFR and PDGFR. Stimulation of TGFβ receptors results in Ser/Thr phosphorylation and activation of transcriptional regulating SMAD proteins. Coupling of EGFR and TGFβ receptors regulates negatively TGFβ signaling by Tyr phosphorylation of SMAD proteins. GPCR, G-protein coupled receptors. PLC gamma, phospholipase Cγ. SMAD are intracellular proteins that mediate signaling from receptors for extracellular TGFβ-related factors.

C. The Ras signaling pathway

The so-called small G proteins are monomeric G proteins composing a superfamily containing polypeptides with molecular masses ranging from 20 to 40 kDa. Presently, >100 small G proteins have been identified in eukaryotes from yeast to humans (359). Complete genome sequence revealed the existence of the Ras subfamily with four members, the Rho subfamily with six members, the Rab subfamily with 11 members, the Sar/Arf subfamily with seven members, and the Ran subfamily with two members.

For the purposes of this review article, we focus on the Ras subfamily. To extend their knowledge on the structure, modes of activation, and action of small G proteins, readers are referred to the excellent review by Takai *et al.* (359).

P21Ras, a 21-kDa protein, is a prototype of the Ras subfamily of small G proteins. These proteins were first characterized in sarcoma viruses as the v-Ha-Ras and v-Ki-Ras (337). Their cellular counterparts, three isoforms—N-Ras, H-Ras, and K-Ras—have recently been identified in humans, and their mutated forms were found to be expressed in human carcinomas (42, 43, 91). Small G proteins, like other G proteins, have their activity controlled by specific interaction with the nucleotides GDP and GTP, and for the GTPase activity, which hydrolyzes bound GTP to GDP and inorganic phosphate (358, 377). As mentioned, GEFs such as Sos and RasGRF stimulate p21Ras activity by promoting the exchange of GDP for GTP. Conversely, GTPase-activating proteins or GAPS accelerate the hydrolytic activity of p21Ras, promoting the formation of the inactive/GDP-bound form of p21Ras (46). Thus, a complete cycle

of activation and inactivation is achieved, making small G proteins, such as p21Ras, to function as molecular switches (Fig. 6).

Another important structural aspect of the small G proteins, which reflects on their activity, is related to posttranslational modifications that permit them to anchor on membranes. These posttranslational modifications are carried out by specific transferases and include farnesylation, palmitoylation, and geranylgeranylation. Sequences containing Cys residues followed by aliphatic or aromatic amino acids (or both) are located in the carboxyl-terminal domains of H-Ras, K-Ras, and N-Ras. The four conserved sequences are as follows: (a) Cys-A-A-X, where A stands for an aliphatic amino acid, and X for any amino acid; (b) Cys-A-A-Leu/Phe; (c) Cys-X-Cys; and (d) Cys-Cys. H-Ras, K-Ras, and N-Ras isoforms display in their structures the Cys-A-A-X sequence. K-Ras is farnesylated at the Cys residue of the sequence, whereas addition of a farnesyl group to the Cys residue is followed by palmitoylation of a second Cys residue in H-Ras and N-Ras (146). After palmitoylation/farnesylation, N-Ras and H-Ras are transported to the plasma membrane *via* the secretory pathway. A polybasic sequence adjacent to the farnesylated Cys residue is essential for integration of K-Ras to the plasma membrane (146, 359).

Not so long ago, active/GTP-bound Ras isoforms were assumed to be located mainly on the plasma membrane in close proximity to the cytoplasmic domain of RPTKs. Only recently, spatial and temporal aspects of p21Ras-mediated signaling have been considered. Response to growth factor–mediated signaling is the same for the three isoforms of p21Ras, which equally bind GTP and integrate to the plasma membrane. However, a delayed and sustained activation of H-Ras, anchored to the membranes of the endoplasmic reticulum and Golgi, was observed in fibroblasts, PC12, and Jurkat T cells (34, 64).

The common characteristic of the mutated *ras* oncogenes is the maintenance of p21Ras in the active/GTP-bound conformation. In most of the cases, this is a result of mutations in the amino acids 12, 13, and 61, which belong to the amino acid consensus sequence responsible for GTPase activity. Thus, activating mutations in p21Ras are prevalent in a wide spectrum of human cancers, ~30% of all human tumors. The tumor type will determine the frequency of p21Ras mutations, with the highest frequencies detected in lung, colon, thyroid, and pancreatic (51).

Upstream signals coming from growth factor receptors are transduced to downstream effectors (46, 359). Cell growth and proliferation in eukaryotic cells is invariably regulated by p21Ras, which occupies a central position in signal-transduction pathways (205). A variety of extracellular stimuli, including polypeptide growth factors, cytokines, hormones, and redox-active species transmit signals through p21Ras. The small G protein mediates its effects on cellular proliferation in part by activating the Ser/Thr protein kinases from the Raf family. C-Raf-1, A-Raf, and B-Raf are recruited from the cytoplasm by the active/GTP-bound conformation of p21Ras. Although c-Raf-1 is ubiquitously expressed and is the most-studied isoform of Raf, all three isoforms of Raf interact with p21Ras. The binding of p21Ras to Raf occurs through interactions with two domains placed at the amino-terminal region of the protein. The known domains are the Ras-binding domain (RBD), amino acids 55–131, and the cysteine-rich domain, amino acids 139–184, that associate with p21Ras, resulting in Raf activation (205, 377).

P21Ras also provides indirect regulatory signals to Raf-1. Recruitment and activation of PI3K by p21Ras with production of inositol triphosphate and other phospholipids can activate Rac, another small G protein. Activated Rac binds and activates p21cdc42/rac1-activated Ser/Thr kinase (PAK), which phosphorylates Raf-1 on Ser338, resulting in activation of the kinase (353) (see Fig. 6). The Src family of PTKs can also contribute to the activation of Raf-1, through phosphorylation on Tyr341 (108). PI3K can also provide an inhibitory signal for Raf-1 through phosphorylation on Ser259 by the Ser/Thr protein kinase Akt (397).

Raf-1–independent but p21Ras-dependent pathways were also documented. These pathways connect p21Ras to the Rho subfamily of small G proteins. Besides being important regulators of actin reorganization, gene expression, and cell-cycle progression, Rho GTPases are important effectors of the function of p21Ras (144). Signaling from p21Ras to Rac involves the protein kinase PI3K in the initial step (366). Initial studies involving the use of wortmannin, an inhibitor of PI3K, and constitutively active mutants of Rac and PI3K revealed that PI3K functions upstream of Rac in the signaling cascades triggered by the activation of RPTKs. In addition, p110, the catalytic subunit of PI3K, is another important effector of p21Ras (310). Consequently, PI3K is positioned downstream with respect to p21Ras and is an important upstream modulator of Rac activity. Although this pathway is the most common, a second pathway independent of p21Ras, involving activated RPTKs, PI3K, and Rac, might exist. This pathway probably relies on the interactions between phosphotyrosines on the RPTK cytoplasmic domain and the SH2 domain of p85, the regulatory subunit of PI3K (278) (see Fig. 6).

D. Signaling by MAP kinases

Downstream, MEK a dual-specificity protein kinase that phosphorylates both Tyr and Thr residues (70), is a common

FIG. 6. Cycle of activation and inactivation of small G-proteins such as p21Ras and activation of Ras signaling pathways. Small G-proteins have their activity controlled by specific interaction with GDP, GTP, and by the GTPase activity that hydrolyzes bound GTP to GDP and inorganic phosphate. GEFs such as Sos and RasGRF, stimulate p21Ras by promoting the exchange of GDP for GTP. GTPase-activating proteins or GAPS accelerate the hydrolytic activity of p21Ras promoting the formation of the inactive/GDP-bound form of p21Ras. Stimulation of RPTKs leads to autophosphorylation of the receptors and phosphorylation of adapter proteins such as Shc. Phosphorylated Shc recruits another adapter protein, Grb2, which recruits the guanine nucleotide exchange factor SoS to the plasma membrane. The complex Grb2/SoS stimulates Ras GDP/GTP exchange. Effectors of the active/GTP-bound form of p21Ras include the protein kinases PI3K and Raf. Downstream PI3K may activate Akt or Rac, whereas Ras-GTP interaction with Raf activates the ERK1/2 MAP kinase signaling pathway.

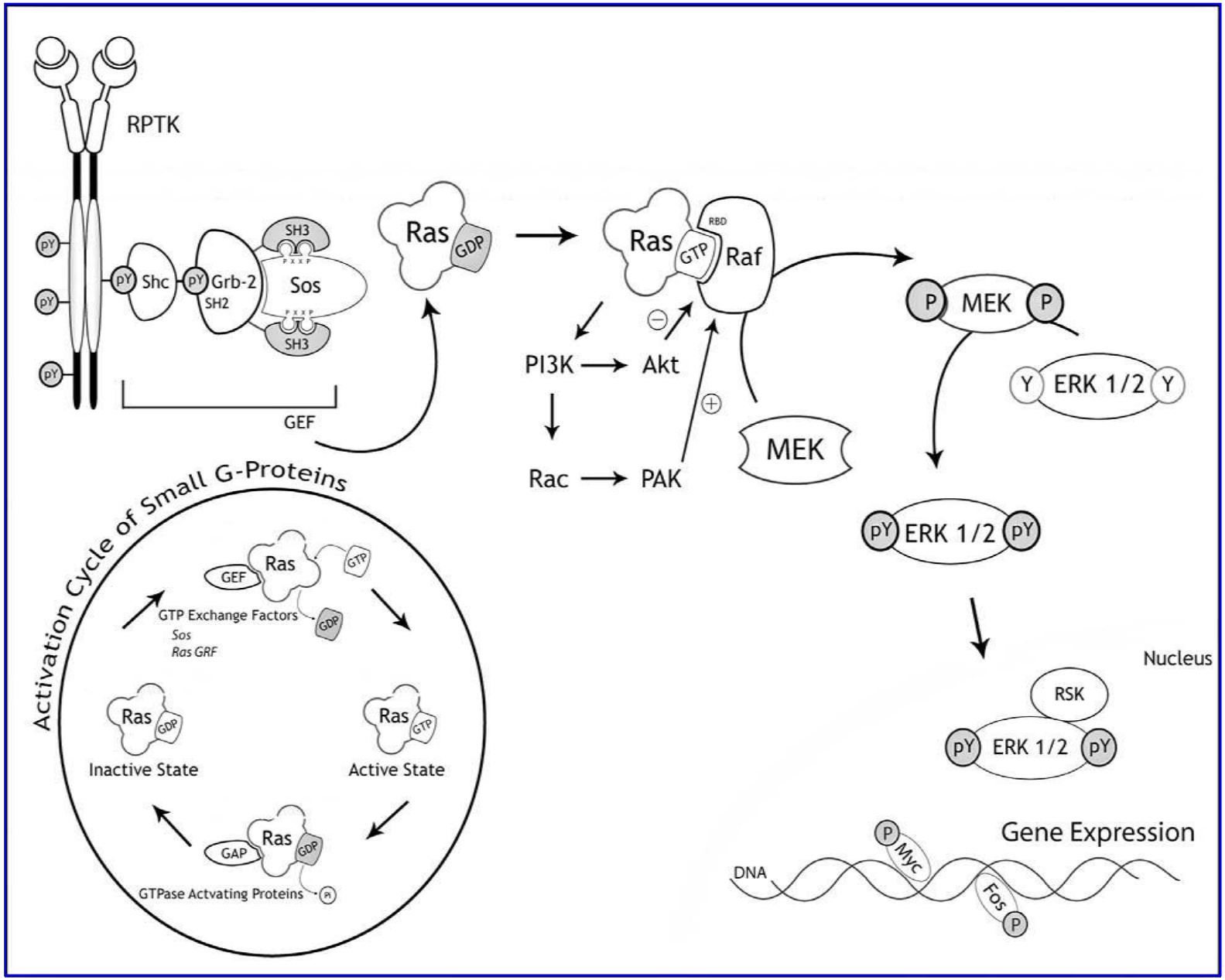
RPTK
pY
Shc
Grb-2
SH2
SH3
Sos
P X X P
GEF
Ras
GDP
GTP
RBD
Raf
PI3K
Akt
Rac
PAK
MEK
P
ERK 1/2
Y
Activation Cycle of Small G-Proteins
GTP Exchange Factors
Sos
Ras GRF
Inactive State
Active State
GAP
GTPase Actvating Proteins
Nucleus
RSK
Gene Expression
DNA
Myc
Fos

FIG. 6.

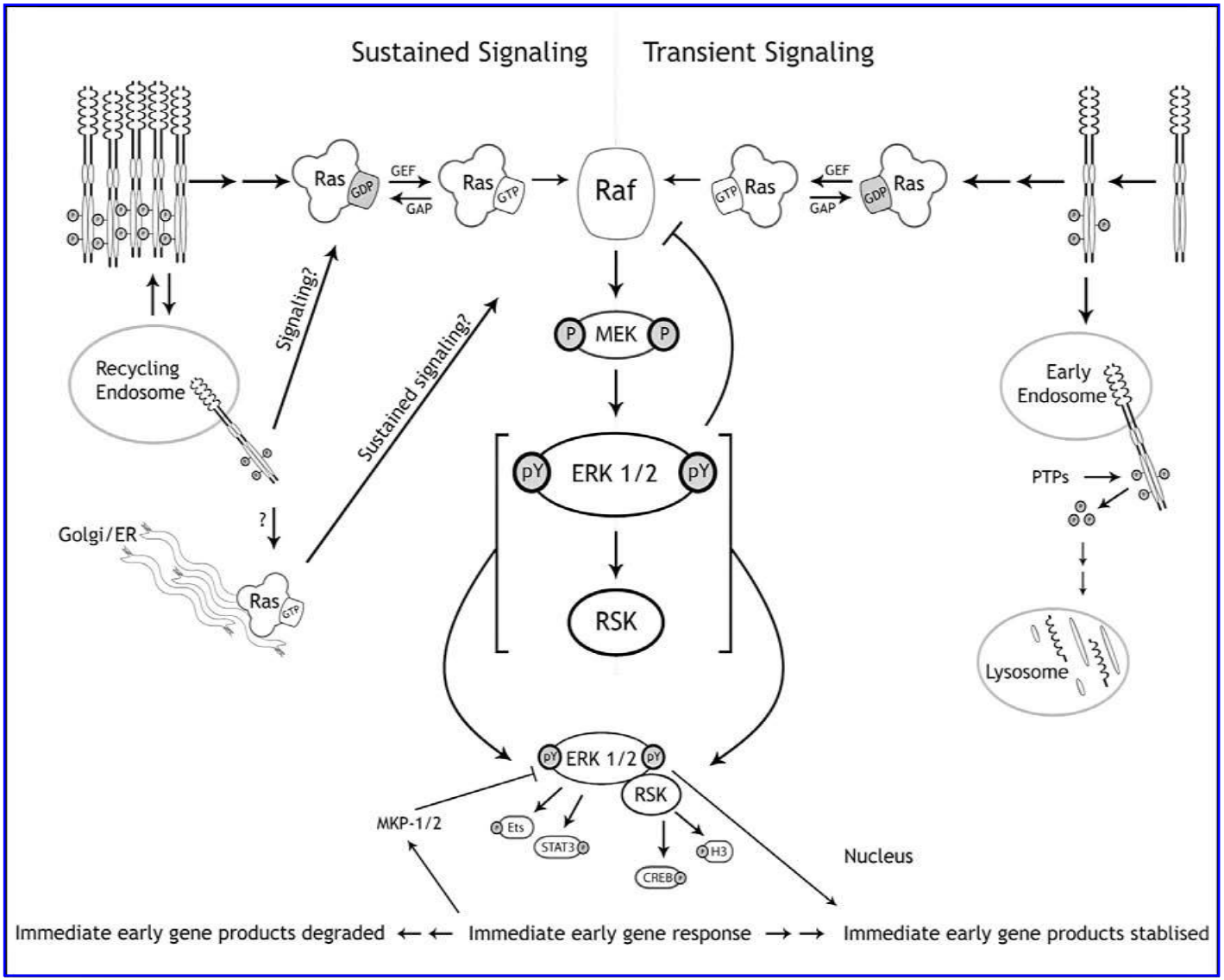
Sustained Signaling
Transient Signaling
Ras
GDP
GEF
GAP
Ras
GTP
Raf
GTP
Ras
GEF
GAP
GDP
Ras
Signaling?
Sustained signaling?
Recycling Endosome
P
MEK
P
Early Endosome
pY
ERK 1/2
pY
PTPs
?
Golgi/ER
Ras
GTP
RSK
Lysosome
pY
ERK 1/2
pY
RSK
MKP-1/2
Ets
STAT3
H3
CREB
Nucleus
Immediate early gene products degraded
Immediate early gene response
Immediate early gene products stablised

FIG. 7.

substrate for the three isoforms of Raf. Raf activates both isoforms MEK-1 and MEK-2, which in turn activate the ERK1/2 MAP kinases. ERK1/2 with 44 and 42 kDa, respectively, are two members of the MAP kinase family of protein serine/threonine kinases that regulate a variety of cellular activities. On activation, the ERK1/2 MAP kinases phosphorylate cytoplasmic substrates such as the ribosomal S6 kinase (RSK) family, which consists of RSK1 to RSK4, and the cytoplasmic domain of the EGFR (269, 297). Phosphorylated ERK1/2 also translocate to the nucleus where, together with nuclear RSK family members, they phosphorylate various transcriptional regulators. Transcriptional activation of immediate early genes and of transcription factors that control the cell-cycle and/or cell-survival results from ERK1/2 kinase activity in the nucleus (270). It was previously assumed that nuclear signaling by ERK would be directly related to signal specificity; however, growth factors that trigger transient and sustained signaling induce similar patterns of immediate early gene expression (109). Signal specificity is derived from a number of factors. In the following paragraphs, we comment on some of them. Early observations gave support to the idea that the two ERKs could phosphorylate specific transcription factors. It was demonstrated that ERK1 was the MAP kinase responsible for phosphorylation of Elk1, and ERK2 phosphorylated preferentially the transcription factor c-Myc (67).

ERK1 phosphorylates the complex ternary factor Elk1 on Ser and Thr residues at the carboxyl terminal domain (369, 385). Elk1 regulates transcription of various genes by autonomous binding to transcriptional regulators such as Ets or as a component of the ternary complex that binds to the serum-responsive element (385, 398). Phosphorylated Elk1 binds to the serum-responsive element triggering the transcription and expression of the protooncogene c-fos. Furthermore, Elk1 is a point of convergence for different signals including signals from oxidants that stimulate the JNK and p38 MAP kinases and may also phosphorylate this transcription factor (89, 398).

Besides spatial regulation of ERK1/2 signaling events, temporal aspects are also crucial for a detailed understanding of the molecular basis of the dynamics of ERK signaling. Although the importance of signal duration and strength is generally accepted, we have only recently progressed in the understanding of how the dynamics of ERK signaling can be interpreted at the molecular level. EGF potently but transiently stimulates the ERK pathway. Sustained activation of the pathway is observed on overexpression of the EGF or insulin receptors (96, 368). Two other contributing factors to signal strength and duration of ERK are the rate and extent of receptor internalization. Beyond termination of the signal, receptor–ligand complex internalization may represent an alternative way of signaling. Signals coming from an internal cell compartment could be transmitted through a connection involving the internalized receptor–ligand complex, p21Ras on the Golgi/endoplasmic reticulum, and cytoplasmic ERK (269) (Fig. 7).

Two other major classes of these kinases have been described, the 46- and 54-kDa c-Jun N-terminal kinases (JNKs), and p38 MAP Kinase. JNKs and p38 MAP kinase mediate signaling cascades initiated by cytokines and stress conditions, including the oxidative stress (125, 237, 251).

Exposure of cells to stress conditions activates the JNK signaling pathway. JNK activation may mediate some of the effects of stress on cells (including apoptosis), or it may represent a survival response stimulated by stress conditions. Thus, the specific role of JNK activation may depend on the cellular context (173). Temporal aspects of JNK signaling are also important as determinants of its role in apoptosis or cell survival. It was demonstrated that sustained activation of JNK is associated with apoptosis, whereas transient activation is associated with survival signaling (61).

Although JNKs are MAP kinases responsive to stress conditions, they may regulate transcription factors also regulated by the ERK1/2 MAP kinases. This is the case for the AP-1 family of transcription factors. The JNKs and ERK1/2 MAP kinases phosphorylate the transcription factors JUN and FOS, members of the AP-1 family, specifically and respectively. JNK phosphorylates JUN on Ser63 and Ser73 residues,and ERK2 phosphorylates FOS on two Serine residues, Ser362 and Ser374. Phosphorylation of FOS will occur only after sustained ERK activation. Inductions of FOS and JUN are normally associated with the mitogenic response, and their superexpressions are associated with cell transformation and carcinogenesis (191).

The signaling cascades for the three MAP kinases very often are overlapping signaling pathways. In many situations, the same or similar signaling molecules are used to promote different functional responses by the MAP kinases signaling pathways. To achieve signaling specificity and simultaneity, formation of protein complexes and scaffolding is an essential step (305). Recent studies provided strong experimental evidence on the role of scaffold proteins in the spatiotemporal regulation of MAP kinases signaling. Scaffold proteins play a major role in regulating signal amplitude and duration of a specific MAP kinases signaling module. This is critical in signaling pathways initiated by stress conditions that will lead to MAP kinases modules that are involved in cell proliferation, differentiation, and cell death (for a recent review, see ref. 94).

FIG. 7. Sustained and transient signaling pathways of activation of the ERK1/2 MAP kinases. After binding of growth factors to their specific RPTKs and activation of p2Ras, Raf proteins are recruited to the plasma membrane and become activated. Raf then phosphorylates and activates the protein kinase MEK1/2, which in turn phosphorylates and dissociates from ERK1/2 MAP kinases. Activated ERK1/2 phosphorylates other proteins at the membrane and in the cytoplasm, including the cytoplasmic domain of the EGF receptor and the ribosomal kinases RSK. Phosphorylated/activated ERK1/2 translocates to the nucleus by an unknown mechanism. RSKs, upon phosphorylation, also migrate to the nucleus, together with the ERK1/2, then both kinases phosphorylate and activate several nuclear targets, including transcriptional regulators (Ets, STAT) and transcription factors (Elk1, c-myc). This results in the induction of immediate early genes. If signaling is sustained, after translation of immediate early genes, these newly translated proteins are phosphorylated and remain active for several hours. If signaling is transient, the products from immediate early genes are unstable and are rapidly degraded by the proteasome.

IV. PROTEIN TYROSINE PHOSPHATASES AND THE REGULATION OF PROTEIN TYROSINE KINASE–MEDIATED SIGNALING EVENTS

A. General description

The complementary function of kinases and phosphatases has been well established and shown to control signaling responses. Kinases determine the amplitude of the signaling response, whereas phosphatases have a role in the control of the rate and duration of the response (367). PTKs and PTPs are involved in the control of cell proliferation, adhesion, and migration.

Protein phosphatases are distributed in structurally distinct families. The serine/threonine phosphatases are complexes of catalytic and regulatory subunits that participate in many signaling pathways. They have two metal ions at the catalytic site of the enzyme that promote the direct hydrolysis of the phosphosubstrate. These phosphatases are targets of several inhibitors that can then be used in the study of phosphorylation-dependent signal transduction. One of these inhibitors is the tumor-promoter okadaic acid. As investigated by Bourdreau *et al*. (44), the Ser/Thr phosphatase PP2A is essential for survival of T leukemia cells. Treatment of these cells with okadaic acid led to a dose- and time-dependent induction of apoptosis. A physical association between the catalytic subunit of PP2A and p38 MAPK in T leukemia cells was observed and prevented activation of the latter. This was reversed by okadaic acid, which allowed phosphorylation of p38 MAPK. In another example (331), inhibition of PP2A by cantharidic acid and okadaic acid caused apoptosis of testicular germ cell tumors through activation of the MEK-ERK signaling pathway that leads to caspase-3–mediated apoptosis. It seems that PP2A, in addition to its participation in the phototransduction pathway and in many other critical cellular processes, exerts antiapoptotic effects on malignant tumor cells. In comparison with this effect, PTPs have both inhibitory and stimulatory effects on cancer-associated signaling processes (284).

The largest family of phosphatase genes encodes PTPs. In the human genome, the number of genes encoding members of the PTP families (107 genes) exceeds that (90 genes) of genes encoding PTKs (244). However, 11 PTP genes are inactive, two dephosphorylate mRNA, and 13 dephosphorylate inositol phospholipids (6). Considering that there are 85 active PTKs, this is equivalent to the 81 protein phosphatases able to dephosphorylate phosphotyrosine, possibly with comparable substrate specificities. The first PTP was purified 10 years after the initial description of PTKs.

PTPs have an active-site motif HCXXGXXRS/T, with cysteine being the nucleophile essential for catalysis (Fig. 8). They are divided into the phosphotyrosine (p-Tyr)-specific phosphatases and the dual-specificity phosphatases (DSPs). The cysteine-based class I PTPs, consisting of 38 strictly tyrosine-specific members (8), all have mouse orthologues. The 61 VH1-like DSPs form the most diverse group concerning substrate specificity (6). The prototypic VH1 DSP is a 20-kDa protein recognized as a virulence factor in vaccinia virus (142). Additional diversity in the family is allowed by alternative promoters and mRNA splicing and by posttranslational modifications. PTPs can act both positively and negatively in the regulation of signal transduction.

Classic PTPs comprise transmembrane receptor–like proteins (RPTPs) and non-transmembrane cytoplasmic PTPs. RPTPs may have cell-adhesion properties in their extracellular domain. Approximately half of them have tandem PTP domains in the intracellular portion. The activity is linked to the membrane-proximal domain called D1. The membrane-distal domain D2 apparently has a role in RPTP dimerization. Cytoplasmic PTPs have regulatory sequences flanking the catalytic site, which may determine the subcellular distribution of the enzymes as well as the substrate specificity. DSPs have little sequence similarity, and, although sharing the same catalytic function of classic PTPs, the active site can accommodate p-Ser or p-Thr in addition to p-Tyr residues. Among the many functions of these enzymes, they turn MAP kinases inactive by dephosphorylation of both p-Tyr and p-Thr, and thus regulate MAP kinase-dependent signaling pathways. In other cases, however, a preferential specificity is found for either p-Ser/Thr or p-Tyr residues in the catalytic activity of DSPs. Small atypical DSPs have been described with functions unrelated to MAP kinases. One of them, PIR1, has RNA 5'-triphosphatase and diphosphatase activities (92).

Dephosphorylation of phosphosubstrates occurs in two steps, consisting of covalent PTP-phosphate intermediate formation and its hydrolysis. The reaction has a high degree of specificity, with PTPs preferentially dephosphorylating subsets of p-Tyr on proteins that carry multiple phosphorylation sites (294). Substrate specificity depends, therefore, on the recognition of regions flanking the PTP active site and on the subcellular compartment that restricts access to the substrate (see Fig. 8).

Classic examples of regulation of cell signaling are PDGF receptor β, which is dephosphorylated by DEP1 and T-cell NT type 2 (cytoplasmic PTPN2). With multiple substrates, PTP1B dephosphorylates insulin receptor, EGFR, and PDGFRs. Phosphocaveolin-1 is also a substrate for PTP1B (225). PTPs antagonize RPTK signaling and modulate their downstream signal transduction. As mentioned, RTKs and PTPs are spatially restricted, being localized to specific subcellular compartments.

Pseudophosphatases have conserved domains of PTPs but have altered residues in the catalytic domain. A glycine residue replaces cysteine in this motif in the inactive STYX, which, however, keeps protein–protein interacting properties of functional relevance (389). An interaction of STYX with the spermatid phosphoprotein CRHSP24 (a 24-kDa calcium-regulated heat-stable protein) is, for instance, important for sperm production (390). D2 domains of RPTPs have point mutations that preclude them from acting as PTP. In analogy, protein kinases may also have both active and pseudokinase domains. For instance, the Janus PTKs (JAKs), which are important regulators of growth-factor and cytokine signaling, have a pseudokinase domain that inhibits the catalytic function. In pathologic conditions, such as that of polycythemia vera, this domain is mutated, resulting in enhanced JAK activity (335).

The *MTM* genes of the human genome can also encode pseudophosphatases. Enzymatically active MTMs can be regulated by inactive MTMs, and their interactions can also determine the subcellular site of the active phosphatase. The lack of catalytic activity of pseudophosphatase MTMs could be due to a negatively charged region close to the catalytic site that

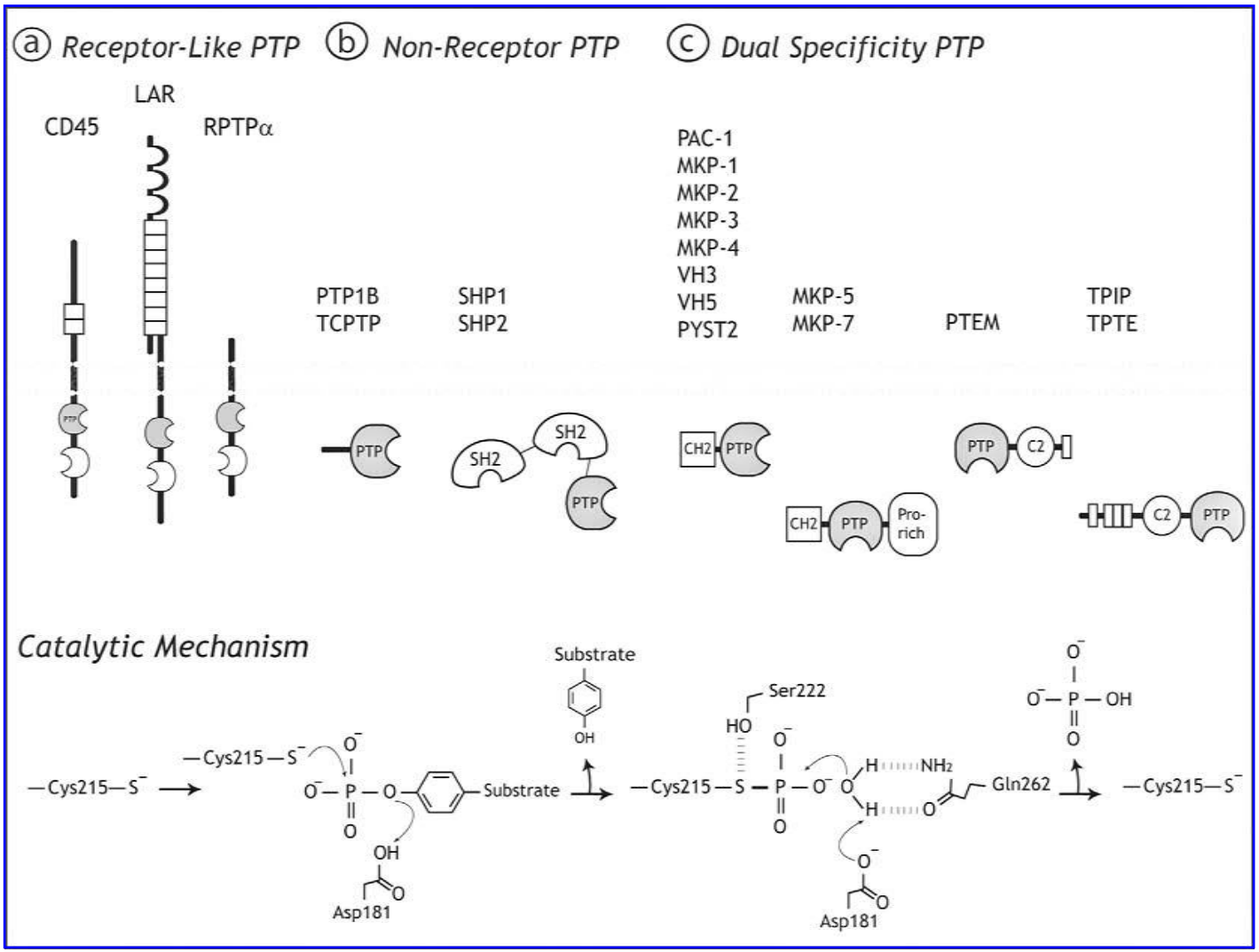

FIG. 8. PTPs and their catalytic mechanism. Protein tyrosine phosphatases can be categorized as receptor-like PTPs, cytoplasmic PTPs, and dual specificity PTPs. All members of the PTP family use the same basic catalytic mechanism. The highly conserved sequence HCXGXXRS/T, which contains the essential nucleophilic Cys residue, forms the base of the active-site cleft and recognizes the phosphate of the substrate. The catalytic mechanism is a two-step mechanism and involves the production of a cysteinyl-phosphate intermediate. In the first step, there is a nucleophilic attack on the phosphate by the sulphur atom of the thiolate anion of the essential Cys residue. This is coupled with protonation of the tyrosyl leaving group of the substrate by the conserved Asp residue. The second step involves the hydrolysis of the phosphoenzyme intermediate, mediated by a conserved Gln residue. In PTP1B, the conserved residues are Cys215, Asp181, and Gln262.

otherwise is positively charged in the active enzyme. The functional role of pseudophosphatases is indicated in the autosomal recessive hereditary motor and sensory neuropathy or Charcot-Marie-Tooth disease, a clinically and genetically heterogeneous disorder of the peripheral nervous system. Mutations in SET-binding factor 2, a member of the pseudophosphatase branch of myotubularins, were identified as the cause of CMT4B2, a severe autosomal recessive demyelinating neuropathy (199). Mutations in SET binding factor 2 lead to shortened or truncated proteins and loss of function.

B. Regulation of receptor protein tyrosine phosphatases

Owing to their critical role in cell signaling, PTPs are controlled by various mechanisms that involve ligand binding, the nature of reactive isoforms, and substrate interaction. Further, some PTPs are regulated by phosphorylation that can either increase or decrease their activity (283). PTPs can also be regulated by proteolytic cleavage associated with degradation or translocation. Reversible oxidation of the cysteine residue of the active site is an important regulation mechanism that is further discussed in the next section. RPTP dimerization is regulated by ligand binding, and in this state, phosphatase activity is attenuated. As opposed to this, RPTKs are activated by ligand-mediated dimerization. Apparently, multiple segments in PTPs, including the D2 domains, contribute to dimerization. Jiang *et al*. (180) generated disulfide-bonded RPTPα homodimers by mutations of single cysteines in the ectodomain region. Expression of wild-type RPTPα and mutants F135C and T141C in RPTPα-null mouse embryo cells increased dephosphorylation and activity of Tyr527 in Src kinase. The P210/211L mutation in the inhibitory wedge of P137C mutant, which did not show increased dephosphorylation, restored its ability to activate c-Src. These data suggested that dimerization could inhibit RPTPα activity *in vivo* (180). They were also consistent with the proposal that dimerization involves interaction of an inhibitory wedge on one monomer with the catalytic cleft of D1 in the other monomer (380).

Inhibitory dimerization involving D1–D1 interactions does not seem, however, to apply to all RPTPs. For instance, this regulation mechanism is unlikely in leukocyte antigen–related PTP (LAR). Also, the structures of D1 and D2 in CD45 preclude this type of regulation because of their spatial orientations (273). In a number of PTPs, the extracellular domains mediate homophilic interactions: PTPμ (*PTPRM*), PTPκ (*PTPRK*) and PTPδ (*PTPRD*).

The regulatory role of the juxtamembrane wedge of the RPTP CD45 was further investigated (158). This protein tyrosine phosphatase has an important role in regulating T-cell and B-cell receptor signaling. The E613R mutation in CD45 resulted in a lymphoproliferative disorder and lupus-like autoimmune syndrome. Hyperresponsive B cells were generated in this mutant displaying activated Ca^{2+} and MAP kinases signaling. Interestingly, natural killer cells from CD45-deficient mice did not secrete cytokine/chemokines after ligand stimulation but had preserved their cytolytic activity (161).

BCR signaling is attenuated in CD45-knockout mice that show hyperphosphorylation of the autophosphorylation site of Lyn. Simultaneously, a decreased phosphorylation of the inhibitory site at the C-terminal is found, with an increased effect of Lyn as an inhibitor of BCR signalling. In the wedge mutant E613R, Lyn is functionally inactive, and this could involve inhibition of dimerization of CD45 or else a restricted interaction with substrates.

CD45 is highly glycosylated and highly expressed in hematopoietic cells. The more abundant *O*-glycosylation and sialylation, as in the RABC isoform, the less efficiency in forming dimers. The smallest-isoform RO dimerizes more efficiently than RABC, resulting in decreased signaling *via* the T-cell receptor. Therefore, dimerization as modulated by sialylation and *O*-glycosylation in the extracellular domain of alternatively spliced CD45 exons may represent a mechanism of termination of the primary T-cell response (391).

The inhibition of RPTP type Z (RPTPζ), after binding of pleiotrophin, is another well-characterized mode of regulation. Oligomerization of this PTP by an artificial dimerizer or polyclonal antibodies against its extracellular region resulted in its inactivation, showing that RPTP-Z is active only in the monomeric form (119). The substrates of RPTP-Z that showed increased phosphorylation were β-catenin and β-adducin, thus implying effects on the cytoskeletal organization. RPTP-Z is expressed in the brain as a chondroitin sulfate proteoglycan. Moreover, RPTP-Z plays an important role in memory formation by modulating Rho GTPase through dephosphorylation at Tyr1105 on p190 RhoGAP (363). Previously, it was shown that age-related impairments of synaptic transmission and long-term potentiation were caused by alterations in the balance of protein kinase and phosphatase activities (169).

Another ligand regulating the PTP LAR positively with high affinity is syndecan, a heparan sulfate proteoglycan (HSPG). The syndecan-LAR complex promoted the growth of presynaptic terminals in *Drosophila* neuromuscular junction and another HSPG, the glycosylphosphatidyl-inositol–anchored glypican, Dallylike, also identified as a high-affinity ligand, inhibited LAR as a controller of synapse morphogenesis and function (183). Apparently, syndecan and Dallylike compete for binding to LAR and modulating PTP activity.

PTPσ of the LAR family of RPTPs has previously been shown to regulate axon growth negatively. Siu *et al*. (340) identified N-cadherin and β-catenin as *in vivo* substrates of PTPσ. In PTPσ knockout mice, elevated tyrosine phosphorylation of N-cadherin resulted in accelerated dorsal root ganglion nerve growth. As a physiologic substrate for PTPσ, N-cadherin mediates inhibition of axon growth.

LAR family of RPTPs has extracellular domains with Ig and fibronectin type III (FNIII) configurations. Whereas syndecan binds to the Ig domain, the extracellular matrix complex laminin-nidogen binds to a splice isoform of LAR that has nine amino acids of the FNIII domain-5 (279). Seven LAR-interacting protein-like genes in humans and in *Caenorhabditis elegans* that form the liprin gene family have also been recognized (334). Liprins are multivalent proteins that may form complex structures. Whereas some liprins have broad mRNA tissue distribution, others are predominantly expressed in the brain. Liprins may localize LAR tyrosine phosphatases to specific sites on the plasma membrane, possibly regulating their interaction with extracellular substrates (Fig. 9).

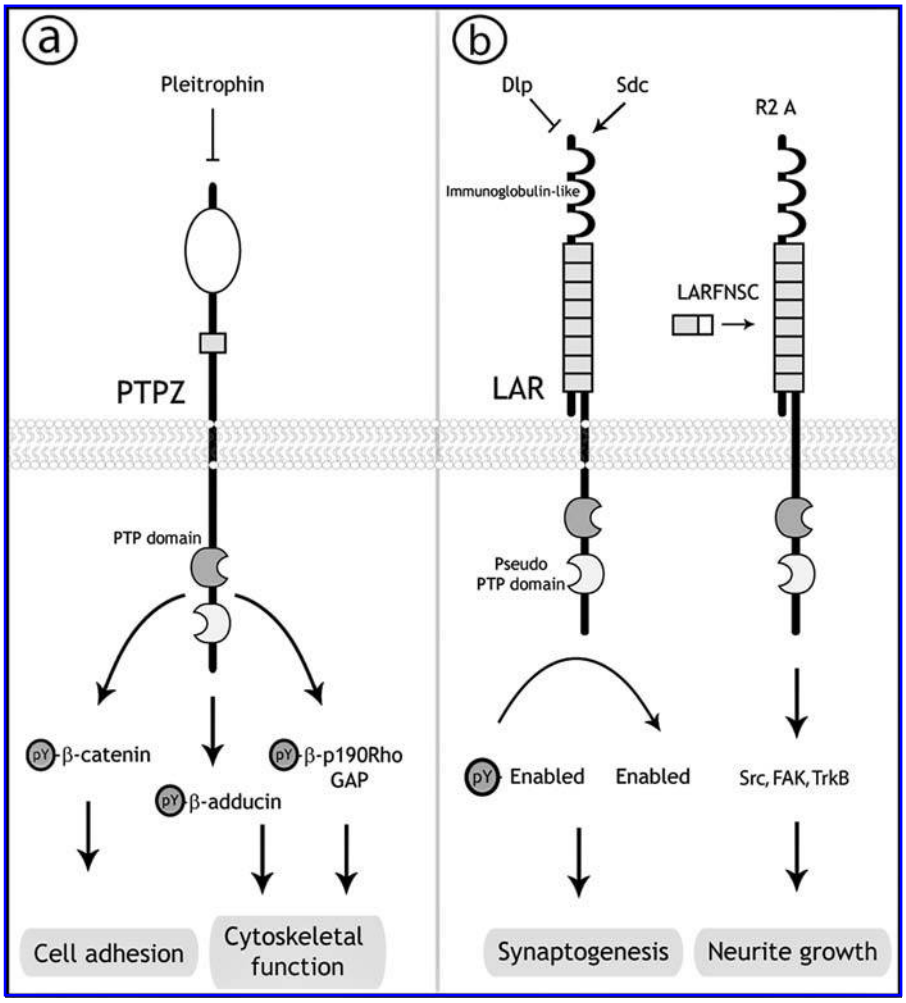

FIG. 9. Regulation of RPTPs by ligands. Examples are given of ligand-mediated responses in receptor PTPs. (**a**) Bonding of pleiotrophin inhibits RPTP-Z promoting Tyr phosphorylation of β-catenin, which regulates cell adhesion, and β-adducin and p190 Rho GAP, which regulate cytosekeletal function; (**b**) RPTP LAR interacts with high affinity with syndecan (Sdc), which promotes PTP activity, but also with GPI-anchored Dallylike (Dlp) protein, which suppresses PTP activity. These ligands regulate Tyr phosphorylation of LAR-associated protein enabled that controls synaptogenesis. A soluble ligand, LARFNSC, binds to homophilic fibronectin domains of LAR, activating signaling pathways that lead to neurite growth.

C. Regulation of cytoplasmic protein tyrosine phosphatases

The cytoplasmic PTPs, TC-PTP or T-cell PTP and PTP1B are homologous although independent enzymes and represent examples of differential regulation and function. PTP1B is a 435-amino-acid 50-kDa protein (59). It has an amino-terminal catalytic domain and two tandem proline-rich motifs that interact with SH3 domains. The human TC-PTP is an intracellular protein with 418 amino acids. TC-PTPa and TC-PTPb are generated by alternative splicing with masses of 45 kDa (mouse and human) and 48.5 kDa (human). PTP1B localizes to the endoplasmic reticulum, whereas TC-PTP has a nuclear localization signal. On mitogenic stimulation, the 45-kDa isoform is found in the cytoplasm (365) and may target proteins associated with the plasma membrane. PTP1B responds to metabolic stress and seems to be regulated by the Y-box–binding protein 1 transcription factor. A correlation was found between the expression of both PTP1B and the Y-box–binding protein 1 in cancer cell lines and type 2 diabetes (120). Both TC-PTP and PTP1B are modulated in cells with high expression of Bcr-Abl, the PTK associated with chronic myelogenous leukemia. Whereas TC-PTP is downregulated, the expression of PTP1B is enhanced. A sequence responsive to Bcr-Abl has been identified in the promoter of PTP1B (121). The expression of PTP1B also is modulated in solid cancers. It can either be decreased, as in esophageal cancer, or increased, as in epithelial, ovarian, and human breast carcinomas (98). The role of TC-PTP in human disease is not clearly defined. It could have a role in cell-cycle progression (45).

PTPs are natural RPTKs antagonists and serve as regulators of both receptor and nonreceptor PTK. Each RPTK is dephosphorylated by a number of PTPs. Dephosphorylation of the receptor can terminate receptor signaling. Otherwise, PTPs can dephosphorylate a subset of tyrosine residues and modulate signaling downstream of the receptor. EGFR and PDGFR are hyperphosphorylated, as stimulated by their cognate ligands in PTP1B-deficient fibroblasts. Similar to PTP1B, TC-PTP controls PDGFRβ phosphorylation on Tyr1021 (294), and thus, it is a negative regulator of PDGF-β–receptor signaling. It seems that PTPs display site selectivity in their action on tyrosine kinase receptors. The regulation of insulin signaling by PTP1B and TC-PTP has been observed in insulin-sensitive tissues, and their activities may have complementary roles. In immortalized TC-PTP($^{-/-}$) murine embryo fibroblasts, insulin-induced insulin receptor (IR) β-subunit tyrosine phosphorylation and Akt acti-

vation were stimulated. Expression of differentially localized variants of TC-PTP (TC45 or TC48) could dephosphorylate IR and downregulate insulin signaling *in vivo* (122).

As mentioned earlier, the activity of PTPs is regulated by oxidation/reduction reactions involving a critical cysteine residue at the catalytic domain. Insulin stimulation that generates a burst of H_2O_2 resulted in transient oxidation and inhibition of PTP1B and TC-PTP (255). In turn, phosphorylation of PTP1B at Tyr66, Tyr152, and Tyr153, also stimulated by insulin, increases the phosphatase activity, thus counteracting the previous effect (81). The phosphoserine content of PTPs may also be regulated by insulin. Possibly, Tyr and Ser phosphorylation regulates PTP1B and TC-PTP activities temporarily, as a function of stimulation.

PTP1B and TC-PTP can negatively regulate signaling molecules of the cytokine pathway. Janus kinases (JAKs) are activated on ligand binding to a number of cytokine and hormone receptors. Some of these are IL-2R, IL-6R, IFN-γR, IFN-αR, and growth hormone receptor. Activated JAKs phosphorylate specific tyrosine residues and recruit STATs. The latter translocate to the nucleus for signaling. PTP1B dephosphorylates JAK2, TYK2, and STAT5. TC-PTP (the 45-kDa isoform) dephosphorylates JAK1 and JAK3 and also STAT1, STAT3, and STAT5, mainly within the nucleus (reviewed in 45).

TC-PTP and PTP1B have a role in erythropoiesis. TC-PTP$^{-/-}$ mice are anemic, and PTP1B dephosphorylates erythropoietin-stimulated erythropoietin receptor, therefore downregulating signaling by erythropoietin *in vitro* (73). Exons 3 and 4 deletion in *TC-PTP* led to mice that, although being born normal, died between 3 and 5 weeks with systemic inflammation. Such a response suggests that TC-PTP has a pivotal role in the control of inflammation (152). B lymphocytes from these animals had impaired response to LPS and to T-cell–dependent stimulation (392).

Cytoplasmic PTPs composed of two SH2 domains and a carboxy-terminal PTP domain are known as SHP-1 and SHP-2. They modulate progenitor cell development, cellular growth, tissue inflammation, cellular chemotaxis, and also control cell survival involving oxidative stress pathways. SHP-1 and SHP-2 are involved in a number of growth-factor and metabolic pathways, being implicated in diseases such as diabetes, neurodegeneration, and cancer. SHP-1 and SHP-2 modulate cellular signaling that involves PI3K, Akt, JAK, signal transducer and activator of transcription proteins (STAT), MAP kinases, and NF-κB (reviewed in 66). SHP-2, is also involved in signaling pathways triggered by cytokines and growth factors, including the MAP kinase, JAK-STAT, and PI3K signaling pathways. It plays an important role in transducing signals relayed from the cell surface to the nucleus, and regulates cytokine and growth factor–induced cell survival, proliferation, and differentiation (275, 303).

D. Protein tyrosine phosphatases as tumor suppressors and oncogenes

The recognition of oncoproteins in the PTK families suggested that PTPs could play a role as tumor suppressors. Overexpression of PTPs reversed PTK-induced transformation (52). PTEN tumor-suppression property results from dephosphorylation of inositol phospholipids, thus regulating cell-survival signaling initiated by PI3K. Other tumorigenic signaling pathways, such as those that involve p21Ras, p53, TOR (target of rapamycin) or DJ1, can also contribute to P13K-PTEN deregulation (76). Inactivating mutations of PTEN with activation of PI3K were followed by activation of p53 in human cells. The loss of PTEN or the oncogenic activation of PI3K, however, increases the selective pressure to mutate p53 (223).

Several potential tumor suppressors have been described among PTPs, including RPTPs, and candidates for a suppressing function have been indicated, based on mutations, particularly of the extracellular segment of the enzyme. Genes encoding PTPs, which are potential suppressors in colorectal cancers, include the greatest number recognized. Other tumor targets were lung and breast cancers, CNS lymphomas, and hepatocellular carcinoma (reviewed in 367). Promoter silencing by epigenetic methylation is another mechanism observed in many established tumor-suppressor genes. Methylation and suppression of the RPTP gene, *PTPRO*, encoding the tumor-suppressor PTPRO, in a variety of solid and liquid tumors has been found. One can speculate whether reactivation of genes silenced by methylation, by using inhibitors of DNA methyltransferases and histone deacetylases, or the direct use of PTPRO, could be a tool in cancer therapy (176). Promoter methylation of PTP genes has been described in lung tumors, hepatocellular carcinoma, and leukemia/lymphoma (reviewed in 367).

Different mechanisms for PTP inactivation in cancer can be summarized as follows:

1. Point mutations in the coding region of PTP genes, as in colon carcinoma and other solid tumors. Several PTPρ mutations affected PTPρ-mediated cell–cell adhesion and inhibition of cell proliferation.
2. Allelic loss of *PTPRJ*, which encodes RPTP DEP1 in various types of carcinomas.
3. Promoter methylation of *PTPN6* (which encodes SHP-1), frequently observed in lymphoma and leukemia (284).

Gain-of-function mutations in some PTPs can, in contrast, generate oncogenes. Mutations in residues in the amino-terminal SH2 domain of SHP-2 lead to cases of the autosomal dominant genetic disorder, Noonan syndrome, whereas somatic SHP-2 mutations are found in hematologic and solid malignancies (193). SHP-2 is at present the only PTP with a proven oncogenic function. Other PTPs were linked to cell transformation, but further studies are needed to establish them as true oncogenes.

PTPs may be expressed in endothelial cells and have a role in angiogenesis. Their possible oncogenic function has stimulated studies on anticancer-drug development. SHP-2 is an obvious target to stimulate the design of selective inhibitors.

V. PROTEIN TYROSINE PHOSPHATASES AS REDOX SENSORS

A. Redox state: GSH and Trx network

The inactivation or activation or both of protein kinases and protein phosphatases is consistently observed in cells exposed

to oxidants or reducing compounds. Signal-transduction pathways are regulated through oxidation and reduction of thiol groups from proteins and enzymes (262, 271, 396).

Early observations described changes on the storage and utilization of glycogen mediated by redox regulation of glycogen synthase and glycogen phosphorylase. Furthermore, purified enzymes from the glycolysis and gluconeogenesis metabolic pathways were found to be redox regulated, implying a redox-based mechanism for regulation of both pathways (396). Moving from metabolism to signal-transduction pathways, accumulating experimental evidence led many investigators to realize that the direction of many cellular processes depends on the redox environment of the cell.

Major reductants, such as GSH and Trx, are associated with their respective oxidized forms to form a linked set of redox couples. The redox environment of a cell reflects the redox state of redox couples, such as the couples GSSG/2GSH and $TrxSS/Trx(SH)_2$.

The redox state is maintained by proteins and nonprotein reducing systems that are fundamentally driven by their intrinsic tendency to donate electrons and to become oxidized (29). When cells, tissues, or an organism are submitted to oxidizing conditions, reducing peptides and proteins, enzymatic, and nonenzymatic antioxidants are oxidized and eventually depleted. Recovery of the reducing capacity of the cells is achieved through cellular redox cycling pathways at the expense of reducing equivalents from NADPH and NADH (319).

Thus, the GSSG/2GSH and $TrxSS/Trx(SH)_2$ redox couples are responsible for the delivery of reducing equivalents from NADPH to peroxiredoxins (Prx) and glutaredoxins (Grx) *via* thioredoxin reductase (TRXR) and glutathione reductase (GR), respectively. Therefore, the redox couples GSSG/2GSH and $TrxSS/Trx(SH)_2$ play critical roles in redox sensing and cycling (234) (Fig. 10).

The glutathione system GSH/GSSG is present in the cytoplasm at high concentrations ranging from 1 to 11 m*M* (129, 342). Measurements of total concentrations of GSH or GSSG or both, as well as the ratio of [GSH]/[GSSG] have been used to estimate the redox environment of a system under investigation. Nevertheless, a measurement of total content of GSH and GSSG in cells would represent an overall redox environment, but the redox environment of each cell compartment would not be represented by these determinations (184, 209, 342, 378). The extracellular levels of GSH are usually 100- to 1,000-fold less than the intracellular levels. Extracellular GSH functions primarily in detoxication processes and also as an antioxidant protecting cells against oxidative injury.

Most of the GSH in cells is found in the cytoplasm, where it is synthesized by two enzymes, γ-glutamylcysteine synthetase and glutathione synthetase (254). However, some organelles have their own GSH pools, independent of the main cytoplasmic pool, such as the nucleus. Nuclear pools of GSH were found to be insensitive to depletion by buthionine-l-sulfoxymine (BSO), an inhibitor of the enzyme γ-glutamylcysteine syn-

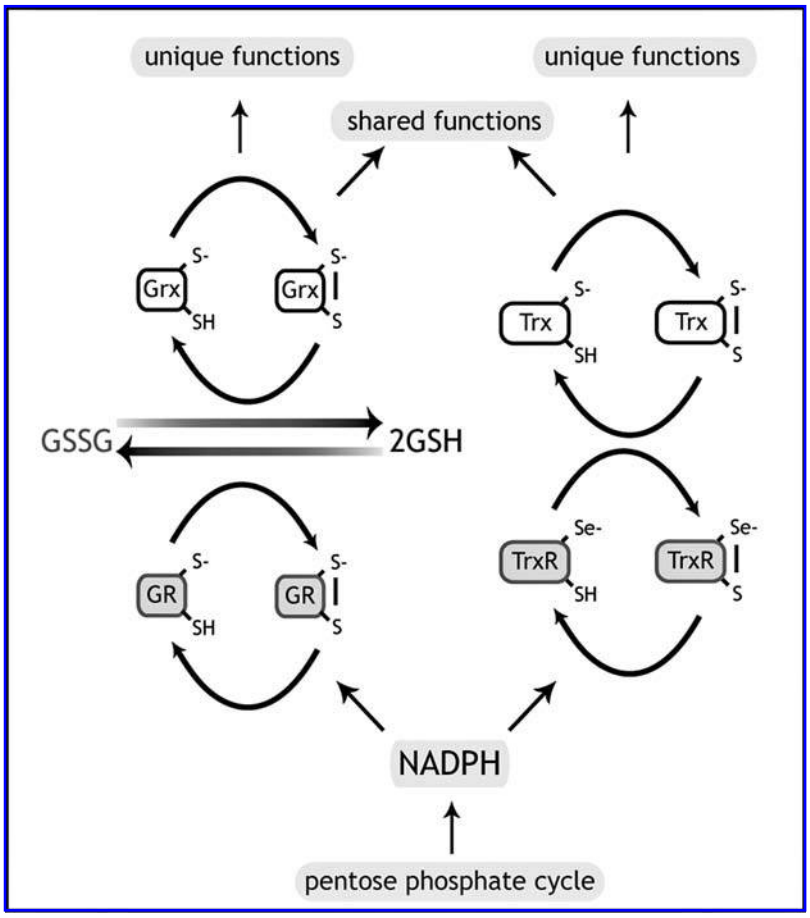

FIG. 10. The redox couples GSSG/2GSH and TrxSS/Trx(SH)2 and the cellular redox environment. The GSSG/2GSH and TrxSS/Trx(SH)2 redox couples are responsible for the delivery of reducing equivalents from NADPH to peroxiredoxins (Prx) and glutaredoxins (Grx) via thioredoxin reductase (TRXR) and glutathione reductase (GR), respectively. Trx and Grx share some responsibilities. However, they also perform their own functions as well.

thetase (137). These findings suggested a specific role for the nuclear pool of GSH in maintaining the redox state of critical protein thiols necessary for gene expression and DNA repair (13, 179). The intracellular ratio of GSH/GSSG, which varies between 30:1 and 100:1, determines the redox environment of the cell. For instance, the endoplasmic reticulum is more oxidizing than the mitochondria and the nucleus, and the ratio of GSH/GSSG in this compartment ranges from 1:1 to 3:1. Therefore, oxidizing or reducing intracellular compartments will display different redox environments when compared with that of the cytoplasm (319). Although present in eukaryotic cells at levels 100- to 1,000-fold less than GSH, the Trx system [$Trx(SH)_2$/TrxSS] also plays an essential role in determining the cellular redox state, acting as a general regulator of signal-transduction pathways (271). Intracellular levels of Trx vary from 1 to 10 μM in mammalian tissue and can reach 15 μM in bacteria (165, 166). The Trx family contains a conserved -Cys-Gly-Pro-Cys- sequence at their active site that is fundamental for reducing activity. Members of the family include cytoplasmic Trx1, the mitochondrial Trx2, and the spermatozoa Trx (167, 234, 258, 347).

Trx is a 12-kDa multifunctional protein that was initially described as an electron donor for methionine sulfoxide reductase and for ribonucleotide reductase. The refolding of disulfide-containing proteins is also facilitated by the thiols from Trx. In addition, Trx has signaling properties that are distinctly determined by its location. In the extracellular milieu, Trx acts as a co-cytokine displaying chemokine-like activity (32). In the cytoplasm, Trx negatively regulates the activity of apoptosis signal–regulating kinase-1 (ASK1) (316). Seminal observations by Saitoh and co-workers (317) demonstrated that only in the reduced state is Trx capable of binding to ASK1, leading to inhibition of its kinase activity. Oxidative-stress conditions lead to Trx oxidation and its consequent dissociation from ASK1, resulting in the activation of the kinase. Accordingly, this dissociation and activation were shown to be prevented by the up-regulation of GSH promoted by NAC (236). Activation of ASK1 leads to the activation of the MAP kinases JNK and p38MAPK (361).

The intracellular Trx is found mainly in the cytoplasm; however, ROS as well as RNS (3, 11, 164, 372) trigger its nuclear migration. In the nucleus, Trx regulates transcription factors including NF-κB, AP-1, p53, glucocorticoid receptor, and polyoma virus enhancer–binding protein 2 (3, 163, 164, 240, 372). Once in the nucleus, Trx displays important roles in cell protection and function. Blocking of Trx nuclear translocation results in increasing susceptibility to ·NO-mediated cell death and prevention of differentiation of neuronal PC12 cells (11, 24). Both events are associated with the ERK signaling pathway. Within this perspective, we recently proposed that the mechanism of Trx nuclear translocation involves the participation of the p21Ras–ERK1/2 MAP kinases signaling pathway (11).

Recently, it was suggested that the direct interaction of Trx with ·NO at the residue Cys69 appears to control its nuclear migration. The authors described a non-nitrosatable mutant form of Trx (Trx-C69S), unable to migrate to the nucleus on exposure to ·NO sources (330). However, early work by Hirota *et al*. (164) demonstrated that mutants of Trx (Trx-C32S/C35S and Trx-C62S/C69S/C73S) migrated to the nucleus in cells exposed to oxidative stress–inducing agents. This would imply that the redox state of Trx is not a requisite for nuclear translocation. Additional studies are needed to elucidate the intriguing mechanism of intracellular trafficking of Trx under redox control. Nevertheless, the mechanism of control of Trx translocation by direct interaction with ·NO may be viewed as specifically related to ·NO-mediated posttranslational modifications that would differ from the translocation mechanism mediated by ROS.

Trx in concert with Trx peroxidase or peroxiredoxin (Prx) has been considered an important reducing system that maintains the redox state by scavenging ROS. Peroxidases of the Prx family use reducing equivalents provided by Trx to reduce H_2O_2 and hydroperoxides (57, 197). However, Prx may have further action on signaling specificity. The Prx isoforms are present in distinct subcellular compartments. Prx I and II are cytosolic and nuclear, respectively; Prx III is restricted to mitochondria; Prx IV is found in the endoplasmic reticulum, lysosomes, and nucleus; Prx V is located in peroxisomes, mitochondria, and nucleus; and Prx VI is found in cytoplasm, mitochondria, and nucleus. The presence of Prx isoforms in distinct compartments of cells may follow a strategy of distribution dictated by the local presence of redox-based signaling complexes. (249, 306, 307). Prx isoforms are grouped within three families, and these families are based on the number and location of the active-site cysteine residues. The Prx I–IV harbor two cysteine thiols; Prx V harbors two atypical cysteine residues; and Prx VI harbors one cysteine at its active site. The reaction of two Cys-Prx isoforms with peroxides converts the reduced Prx in sulfenilated Prx at the amino-terminal Cys residue. The sulfenic intermediate then reacts with carboxy-terminal Cys of a second enzyme, forming a disulfide homodimer that is further reduced by Trx. Sulfenil intermediates may be further oxidized to sulfinic acid. Sulfinil Prx, however, cannot be reduced by reductants such as Trx, but only by a high substrate-specific reductase for sulfinilated Prx: the sulfiredoxin (33, 178). The hyperoxidized Prx (Cys-SOOH) are inactivated, and restoration of their activity by sulfiredoxin requires ATP hydrolysis (224). This mode of control of Prx activity has been suggested as a built-in mechanism to prevent dampening of the H_2O_2-mediated signaling (307).

The reducing system of the Trx superfamily members, the glutaredoxins (Grx), uses reduced GSH as substrate in a coupled system with GSH reductase and NADPH. Three Grx have been identified in mammalian cells: the dithiol and cytosolic Grx1; the mitochondrial and nuclear Grx2; and a monothiol Grx5 (234, 386). Grx1 catalyzes the reduction of protein disulfide *via* a disulfide exchange reaction by using the active site Cys-Pro-Tyr-Cys with a dithiol mechanism involving both active-site thiols (136). The particular ability of Grx1 is observed in the reduction or formation of protein-S-S-glutathione mixed disulfide (glutathionylation/deglutathionylation) through a monothiol-based mechanism (336). By this action, Grx1 is implicated in the regulation of redox signaling through mediators such as p21Ras and nuclear factor 1 (2, 26). In the mitochondria, Grx2 may be a substrate for Trx reductase, thus behaving like the protein Trx (182). Under oxidizing conditions in which GSH is consumed, the Grx2 will not be reduced by GSH, a situation that may trigger apoptosis. In this context, Grx2 reduction by Trx reductase may rescue cells from apoptosis (194).

Grx5 has been implicated in the iron–sulphur assembly, a

key step for heme biosynthesis and erythrocyte differentiation (386). Once matured, the erythrocyte life span is now predicted by the levels of Prx II, and therefore, by the reducing capacity of the red blood cells (228). As in this example, a number of possibilities of specific actions ascribed to GSH/Grx and Trx/Prx systems have been described (234). These systems may work in concert to compensate for their function or to participate in overlapping reactions. Actions may range from the fine-tuned redox signaling to the control of the redox state in cells exposed to conditions of severe oxidative stress.

The redox environment may determine the final cellular outcome regarding cell proliferation, cell differentiation, or cell death (319). A cellular reducing environment provided by high concentrations of thiols stimulates the proliferation of tumor cells (113). In contrast, thiol depletion or increasing concentrations of lipid hydroperoxides (9), quinones (99, 264), reactive aldehydes, and dithiocarbamates increase the intracellular concentrations of ROS that can induce apoptosis.

Changes in the redox environment were observed during phorbol ester–induced differentiation of THP-1 cells. A predominantly oxidizing environment was generated by preincubation of cells with the GSH-depleting compound BSO. The expression levels of p21waf1, an inhibitor of cyclin-dependent kinases, and of TNF-α, were elevated on GSH depletion. In addition, the phosphotyrosine levels of the cytoplasmic PTK Pyk2 were upregulated by pretreatment of the differentiating cells with BSO. Conversely, Pyk2 tyrosine phosphorylation levels were downregulated if the differentiating cells were pretreated with *N*-acetylcysteine (NAC), a reducing compound that induces GSH synthesis (87). Therefore, changes in the redox environment toward oxidizing conditions switch on the differentiation process, as predicted by Schafer and Buettner (319).

B. Redox environment and signal transduction

As mentioned earlier, both oxidative and nitrosative stresses can alter the cellular redox environment. Changes in the cellular redox environment will bring about changes in the thiol status of the cell. ROS and RNS, such as H_2O_2, O_2^-, and ·NO, can promote the oxidation of the thiol group of Cys. The thiol group of Cys can be oxidized to sulfenic acid (PSOH), disulfide (PSSP), sulfinic acid (PSO_2H), or sulfonic acid (PSO_3H). Sulfenic acids and disulfides are readily reduced; sulfiredoxin reduces sulfinic acid, whereas sulfonic acid is an irreversible state of thiol oxidation (160) (Fig. 11).

ROS potentially induce the initial formation of sulfenic acid, the first intermediate of oxidation, which then reacts with a thiol, forming an intramolecular or intermolecular disulfide. The most common disulfides formed in the cells are the so-called mixed disulfides that are formed with Cys or GSH. When GSH forms the disulfide with the protein thiol, the protein is glutathionylated, which is considered a form of protection against further oxidation (68, 113).

Another important thiol oxidation product is the nitrosothiol (SNO). Nitrosothiols are formed through the reactions of thiols with ·NO, the nitrosonium cation -NO^+, and higher nitrogen oxides such as N_2O_3. Alternatively, ·NO can react with thiyl radicals to form SNO, or SNO can be obtained through transnitrosation by low-molecular-weight nitrosothiols such as *S*-nitrosoglutathione (GSNO). Although enzymatic mechanisms of catalysis of S-nitrosation are still not understood (259), cellular reductants such as Trx and GSH can reverse this posttranslational modification. In addition, Trx was shown to catalyze nitrosation as well as denitrosation of proteins (259, 276). Well-characterized determinants of specificity of protein S-nitrosation include the following:

1. The presence of an acid–base motif, consisting of Asp/Glu and Arg/His/Lys residues in the vicinity of the target cysteine residue;
2. Hydrophobic compartmentation, which allows S-nitrosation by ·NO and higher nitrogen oxides (NO_2, N_2O_3, N_2O_4). Whereas hydrolysis of nitrogen oxides is inhibited in hydrophobic compartments, S-nitrosation based on the direct reaction between ·NO and the thiolate anion is favored in such compartments;
3. The availability of proteins that are capable of capturing ·NO, such as Trx. This implies that the specificity for nitrosation may be determined by the capacity of S-nitrosated Trx to interact with protein targets for nitrosation (260).

Based on these characteristics, S-nitrosation has been considered the prototype of a redox-based posttranslational modification with signaling properties (extensively reviewed in 160).

Changes in the cellular thiol status initiate redox-based signaling cascades with consequences on signal-transduction events. Events such as ligand binding to its cognate receptor, DNA binding to transcription factors, and nuclear translocation of signaling proteins have been shown to be under redox control (5, 262, 333).

Until recently, phosphorylation of proteins and oxidation–reduction of protein thiols were considered separate and independent pathways for cellular signaling. However, accumulating experimental evidence favors a closer relation between the two pathways. We start to examine this evidence by looking at the redox regulation of protein tyrosine phosphatases (PTPs). As mentioned earlier, all members of the PTP family are characterized by the presence in the active site of a highly conserved sequence HCXXGXXRS/T, which contains a Cys residue that is essential for catalysis. Owing to the unique environment of the PTPs active site, the essential Cys residue of the conserved sequence displays a low p*K*a and is predominantly present as a thiolate anion at physiologic pH. This thiolate anion is highly nucleophilic and consequently very susceptible to oxidation. Indeed, oxidation or site-directed mutagenesis of the essential Cys residue renders all PTPs catalytically inactive (115) (see Fig. 11).

Elevated levels of oxidants will cause changes in the redox environment with consequences on cell signaling. The oxidant H_2O_2, a two-electron reduced byproduct of O_2 metabolism, has been considered a toxic waste for many years. However, it is now clear that H_2O_2 is produced by mammalian cells, performing crucial roles in signaling processes associated with cell proliferation, differentiation, and migration (349). Pioneer studies, involving the addition of exogenous H_2O_2 to cultured hepatoma cells H-35 and Fao, showed increasing levels of phosphorylated Ser, Thr, and Tyr (150, 207). Oxidative inhibition of phosphatases was suggested to be operative in both situations; however, at that time, the mechanisms underlying such redox-signaling properties were not understood. Inhibition of

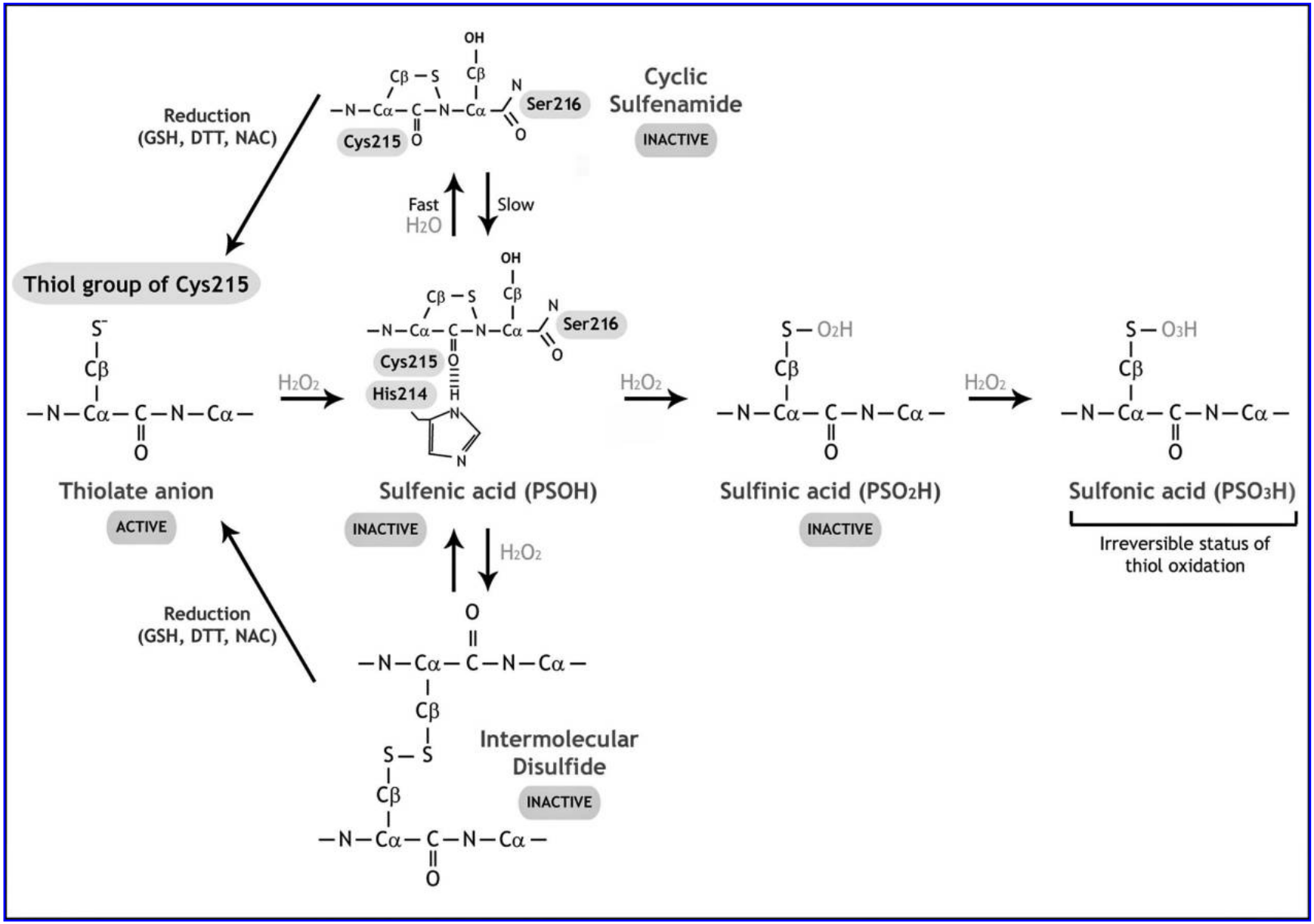
Reduction
(GSH, DTT, NAC)
Cyclic
Sulfenamide
Ser216
Cys215
INACTIVE
Fast
H2O
Slow
Thiol group of Cys215
H2O2
His214
Thiolate anion
ACTIVE
Sulfenic acid (PSOH)
INACTIVE
Sulfinic acid (PSO2H)
INACTIVE
Sulfonic acid (PSO3H)
Irreversible status of
thiol oxidation
Reduction
(GSH, DTT, NAC)
Intermolecular
Disulfide
INACTIVE

FIG. 11.

PTP activities by oxidants can result in increased levels of proteins phosphorylated on tyrosine. Therefore, a dynamic relation between tyrosine phosphorylation/dephosphorylation was suggested.

In 1982, Swarup and co-workers (229, 312) were the first to show the inactivation of PTPs on exposure of cells to increasing concentrations of sodium vanadate. Vanadate, the pentavalent state of vanadium, undergoes intracellular redox cycling and is rapidly reduced to tetravalent vanadium by GSH and Cys. Although oxidative inactivation of PTPs by vanadate was suggested, later observations demonstrated that vanadate inhibits PTPs by mimicking the tetrahedral geometry of phosphate ion. It was shown that vanadate ion formed a covalent linkage with Cys12 at the active site, exhibiting a trigonal bipyramidal geometry. These studies gave support to the conclusion that vanadate inhibits PTPs by acting as a transition-state analogue inhibiting PTPs in a reversible manner (394). Early findings on the strong inhibitory effects of the combination of vanadate and H_2O_2 were followed by the description of peroxovanadium compounds (151, 300). These compounds derive from the reaction of vanadate salts with H_2O_2 or organic peroxides and irreversibly oxidize the critical Cys residue at the active site of PTPs (300) (see Fig. 11).

PTPs were first examined as redox sensors in signaling events by treatment of murine fibroblasts with the thiol-oxidizing probe diamide (208). Diamide promoted the oxidation of the PTP essential Cys residue to sulfenic acid. This also caused inhibition of PTP activities, although this inhibition was transient and was reversed by incubation of fibroblasts stimulated with EGF and maintained in high-glucose medium, or with 2-mercaptoethanol (265).

Production of ROS and RNS by eukaryotic cells can be obtained in cellular and subcellular systems. Sources include the mitochondrial respiratory chain, the oxidative metabolism of fatty acids in the peroxisomes, oxidative reactions catalyzed by the cytochrome P450 family, the respiratory burst on phagocytes, and the ·NO synthases (NOS).

Biologic generation of ·NO comes from the oxidation of L-arginine catalyzed by three different isoforms of NOS. Physiologic levels of ·NO are primarily generated by the constitutive isoforms of NOS, the neuronal isoform (NOS1), and the endothelial isoform (NOS3). Both isoforms were structurally characterized as cytochrome P450 reductase–like enzymes and can produce large amounts of ROS when deprived of their critical cofactor tetrahydrobiopterin or their substrate L-arginine (48). Deprivation of either the cofactor or the substrate leads to an NOS uncoupling, in which electron flow through the enzyme results in reduction of O_2 at the prosthetic heme site rather than formation of ·NO (220, 301, 383).

Higher levels of ·NO are produced by the inducible isozyme, NOS2, which plays a major role in mediating host inflammatory responses. Under pathologic conditions, expression of this enzyme is transcriptionally upregulated severalfold by a variety of inflammatory mediators (203).

Regarding the biologic sources of ROS, ~0.1% of the O_2 consumed by the mitochondrial respiratory chain can be converted into H_2O_2 (172). Apparently, all the mitochondrial H_2O_2 comes from spontaneous or catalyzed dismutation of O_2^-, the one-electron reduction product of O_2 (47). In mitochondria, the use of mathematical models and rate constants obtained experimentally for generation and degradation of H_2O_2 resulted in a concentration value of 0.04 μM H_2O_2 (9).

The presence of catalase in the peroxisomes reduce the levels of H_2O_2 so that their concentrations may reach levels as low as 0.001 μM. The physiologic relevance of such low levels of H_2O_2 in this organelle is questionable (31).

The metabolism of xenobiotics by the isoforms of the cytochrome P450 uses NADPH and an organic substrate. The substrates receive electrons from the cytochrome and transfer them to O_2, generating O_2^- and H_2O_2 (206).

The most extensively studied cellular source of H_2O_2 is the neutrophil. Neutrophils belong to the immune system, and their primary duty is to defend a host against infections by killing and engulfing microorganisms. The defense system relies on the NADPH oxidase (NOX) enzymatic complex to exert microbial killing (18, 19). In response to appropriate stimuli, neutrophils generate O_2^- and H_2O_2 by a signaling cascade that involves the assembly of cytosolic and membrane-associated components to form the active NOX enzymatic complex, also known as NOX2. This complex consists of a $gp91^{phox}$ catalytic subunit bound to five other regulatory subunits, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and the small G protein Rac. The catalytic subunit is a transmembrane protein, which contains an NADPH binding site, and FAD bound to its carboxy-terminal cytoplasmic domain. The amino-terminal domain on $gp91^{phox}$ contains a binding site for two iron-heme prosthetic groups and consists of six membrane-spanning helices. The electron flows from the NADPH bound to the cytosolic domain, through FAD, the heme prosthetic groups, and reaches the O_2 molecule, generating O_2^-. Conversion of O_2^- to H_2O_2 is readily achieved by the activity of the extracellular form of superoxide dismutase (111) (Fig. 12).

Physiologic levels of H_2O_2 can be mimicked by exogenous administration of the oxidant to cultured cells. It is important to emphasize that H_2O_2 permeates through the plasma membrane, and inside the cell, peroxidases and catalases readily metabolize it. Therefore, after exogenous additions of H_2O_2 to cultured cells, a rapid equilibrium is established, and the intra-

FIG. 11. Reversible and irreversible oxidation of the active site Cys residue on PTPs. Oxidation of the active site Cys residue prevents its nucleophilic function, thereby inhibiting PTP activities. In physiological conditions, oxidation of the Cys residue is reversible because of the rapid reduction of the sulfenic acid or disulfide forms of the oxidized Cys. The sulfenic acid intermediate produced in response to physiological oxidation is rapidly converted into a sulfenamide species, in which the sulfur atom of the catalytic Cys (Cys215) residue is covalently linked to the main chain nitrogen atom of an adjacent Ser (Ser216) residue. Under conditions of oxidative/nitrosative stress, higher levels of oxidation of the thiol group—sulfinic and sulfonic acids—are dominant. If oxidation levels of the catalytic Cys residue of PTPs result in the formation of sulfonic acid, inhibition of PTPs is irreversible. (The residues His2114, Cys15, and Ser216 for PTP1B are represented in the figure).

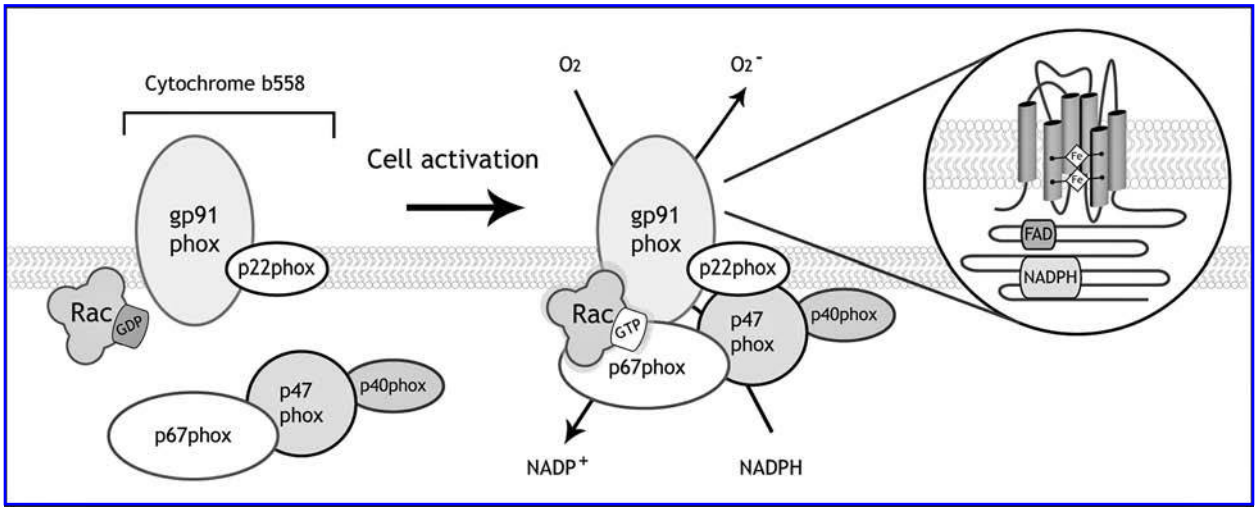

FIG. 12. Activation of the phagocytic isoform of the NADPH oxidase complex. Activation of the NADPH oxidase complex of phagocytes involves phosphorylation of the regulatory cytoplasmic components p47phox, p67phox, and p40phox. Phosphorylated components translocate to the plasma membrane to interact with flavocytochrome-b558, which is composed of gp91phox and p22phox. Activation of the complex also involves guanine nucleotide exchange on the GTP-binding protein Rac stimulated by guanine nucleotide exchange factors.

cellular concentration of H_2O_2 will be ~10-fold less than the extracellular concentration (349).

The reversible and consequently physiologically relevant inhibition of total PTP activities was initially observed in fibroblasts treated with exogenous H_2O_2 (352). Spontaneous recovery of PTP activity was observed in cells maintained in complete medium in the absence of H_2O_2. Preincubation of the cells with BSO, which depletes GSH in the cell, retards the recovery of PTP activity, implicating GSH levels and the redox environment in the process. Conversely, thiol-reducing compounds were shown to reverse the H_2O_2-mediated oxidation of the active-site Cys residue on PTPs. Reversible oxidation of PTP1B by H_2O_2 was demonstrated in EGF-stimulated A431 cells or insulin-stimulated 3T3-L1 adipocytes, and this inhibition was prevented by catalase. It was concluded from these observations that H_2O_2 is required for productive RPTK-initiated signal transduction (226, 238, 239).

In all studies, as discussed earlier, oxidation of Cys to sulfenic acid by H_2O_2 is reversible and thus has the potential to form the basis of a mechanism for reversible regulation of PTP activity. Oxidative alterations of reactive Cys residues primarily lead to the formation of sulfenic derivatives that, in the presence of GSH, are S-glutathionylated and converted to a mixed disulfide. Thus, S-glutathionylation plays an important role as a first line of defense against the irreversible oxidation of the Cys residue to sulfonic acid (68, 113). Despite all considerations, the reversible aspect of H_2O_2-mediated inactivation of PTPs is still debatable. The efficiency and specificity of H_2O_2 regarding the reversible oxidation of Cys215 essential for PTP1B activity has been questioned. Based on the kinetics and structural data, Barrett *et al*. (28) showed that O_2^- is more efficient and chemically a more-specific oxidant than H_2O_2 for inactivating PTP1B. These authors showed that oxidation and inactivation of PTP1B by H_2O_2 produced oxidized Met residues with a lower degree of reversibility. In contrast, if O_2^- was the oxidant, the Cys215 sulfenic acid derivative was S-glutathionylated by GSH. S-glutathionylated PTP1B was then reactivated by thiol-reducing compounds (28).

Further studies by a number of groups focused on redox-mediated reversible inhibition of total cellular PTP activities or on specific PTPs such as SHP-1 PTP, SHP-2 PTP, the lipid phosphatase PTEN, and low-molecular-weight PTP (55, 227, 256, 379).

Although the catalytic Cys residue is a general feature of all classes of PTPs, differences in oxidation sensitivity of this particular Cys residue were observed (139, 379). It has been proposed that the catalytic Cys from the low-molecular-weight PTP, and from the lipid phosphatase PTEN, are protected from irreversible oxidation by disulfide bond formation with vicinal Cys residues (55, 227). However, the tyrosine-specific PTPs such as PTP1B do not display vicinal thiols at their active sites. To prevent irreversible oxidation of the catalytic Cys in these enzymes, an alternative mechanism was recently described (318). In this mechanism, the sulfenic acid intermediate produced after exposure of PTP1B to physiologic concentrations of H_2O_2 is rapidly converted into a sulfenamide species in which the sulfur atom of the catalytic Cys (Cys215 in PTP1B) is covalently linked to the main-chain nitrogen atom of an adjacent Ser residue (Ser216) (see Fig. 11).

Differences in the oxidation sensitivity of the catalytic Cys were also found among SH2-containing PTPs and regular PTPs. SHP-1 and SHP-2, which are structurally homologous PTPs that possess tandem SH2 domains, were found to be quite resistant to oxidation, as compared with the catalytic Cys on PTP1B (379).

Antimicrobial responses in neutrophils are thought to depend on tyrosine phosphorylation of several proteins induced by soluble and particulate stimuli (20). Inhibition of the leukocyte PTP CD45, after stimulation of the enzymatic complex NADPH oxidase with production of ROS in stimulated neutrophils, re-

sulted in increased levels of tyrosine phosphorylation in these cells (112). Inhibition of CD45 was prevented by diphenylene iodonium, an oxidase inhibitor, and by the thiol-reducing compounds, NAC and dithiothreitol (112). Our studies have emphasized the importance of the redox environment on the redox-based regulation of PTP activities in neutrophils. Incorporation of vitamin E into isolated human neutrophils inhibited oxidant production by the cells stimulated with soluble (PMA) or particulate stimuli (opsonized zymosan). PTP activities were downregulated on neutrophil stimulation with PMA or opsonized zymosan. Incorporation of vitamin E completely reversed the inhibitory effects on PTP activities by PMA. Partial recovery of PTP activities was observed in opsonized zymosan-stimulated neutrophils enriched with vitamin E (58).

Oxidized low-density lipoprotein (oxLDL) constitutes an important pathophysiologic stimulus for the production of ROS and RNS in endothelial cells (348, 399). Through a still unknown mechanism, oxLDL can modulate the cellular sources of $\cdot NO$ and O_2^-/H_2O_2, namely the enzyme eNOS, and the vascular NOX (117). Thus, changes in the redox environment promoted by the incubation of rabbit aortic endothelial cells with oxLDL result in the inhibition of PTP activities (118).

Ionizing radiation, specifically the ultraviolet (UV) radiation, can be a source of oxidants in aqueous media. Although this radiation does not have sufficient energy to ionize water molecules, it can cause homolytic fission of H_2O_2 and hence generate OH. radicals, potent oxidants. UV radiation targets several biomolecules as part of its signaling responses. For instance, the RPTKs EGFR and PDGFR are autophosphorylated within seconds of UV irradiation of cells in culture. Several components of signal-transduction pathways, Grb2, PLC-γ, and Shc, which contain SH2 domains or PTB domains, associate with the receptors in UV-irradiated cells (71, 159, 314). UV-stimulated tyrosine phosphorylation levels in RPTKs may result from oxidative inactivation of associated PTPs. The fact that excess NAC prevents the inactivation of PTP SHP-1 associated with PDGFR in A431 cells overexpressing the PTP is a strong indication for the occurrence of oxidative enzyme inactivation. Hydroxyl ($OH\cdot$) radicals generated from UV radiation, absorbed in the aqueous medium where cells are maintained, could oxidize the critical Cys residue present in the catalytic domains of all PTPs (140).

T lymphocytes are very sensitive to UV radiation. On exposure to UV, they become insensitive to mitogens and show markedly reduced rates of proliferation (400). Intracellular calcium levels were elevated in the Ramos Burkitt lymphoma cell line and in the leukemic T-cell line Jurkat exposed to UV radiation. In addition, UV rapidly induces tyrosine phosphorylation of PLC-γ and of its SH2-associated proteins in these cells (324). Later findings by the same group described the UV-induced tyrosine phosphorylation of the T-cell PTK ZAP-70. UV-induced tyrosine phosphorylation of ZAP-70 was not observed in $CD45^-$ T cells, suggesting that tyrosine phosphorylation levels on ZAP-70 are redox regulated through the RPTP CD45 (325).

As discussed in the previous section, differently from the cytoplasmic PTPs, RPTPs display a catalytic domain followed by a regulatory domain. Redox regulation of RPTPα mediated by H_2O_2 or UV radiation essentially involves the oxidation of the essential Cys residue in the regulatory domain (295, 296). Additional studies of RPTPα also demonstrated that under oxidative stress conditions, the oxidation of the essential Cys residue on the regulatory domain is followed by a conformational change that modulates the enzyme activity (36). Conversely, RPTPα, which has also been associated with integrin-mediated signaling through dephosphorylation of Tyr527 on Src kinase, apparently is not regulated by redox signaling during cell adhesion (128, 351).

Another of redox regulation of RPTP activities is their susceptibility to H_2O_2 (139). By using a specific antibody to recognize the oxidized form of PTPs, Groen *et al*. (139) found almost complete oxidation of the RPTP LAR catalytic domain with as little as 60 μM H_2O_2. By contrast, the same authors described that the catalytic domain of RPTPα exhibits only moderate susceptibility to H_2O_2.

The evidence discussed leads to the assumption that PTPs may function as intracellular redox sensors, and their transient inactivation reflects positively on the intracellular phosphotyrosine levels. However, changes in the redox environment will have consequences on other signaling molecules, such as protein kinases and G proteins, with changes in the tyrosine phosphorylated proteins. In the following sections, we examine these other possibilities.

VI. GROWTH FACTORS AND ADHESION SENSORS AS GENERATORS OF OXIDANTS

A. Growth factor receptors and the generation of reactive oxygen species

Oxidants can stimulate tyrosine phosphorylation of cytoplasmic PTKs and RPTKs without affecting PTPs. Changes in the redox environment may affect RPTKs, leading to activation of the receptor independent of the presence of its ligand. Bauskin *et al*. (30) described the redox-mediated activation of the intracellular receptor tyrosine kinase Ltk, located in the endoplasmic reticulum of B-lymphocytes. A putative redox sensor was described as a Cys-rich sequence next to the transmembrane domain of the Ltk receptor. On incubation with diamide or iodoacetamide in the absence of a receptor ligand, the Ltk receptor was phosphorylated on tyrosine (30).

Hydrogen peroxide strongly stimulated EGFR tyrosine phosphorylation levels on carcinoma cell lines overexpressing the EGFR (123). In addition, the combined action of oxidants and growth factors leads to further enhancement on the RPTK phosphotyrosine levels, above the levels detected after incubation with the growth factor alone (63, 298). Thus, oxidants must be seen as major players in signal-transduction pathways leading to cell proliferation or cell survival.

Physiologic levels of ROS and RNS stimulate proliferation or promote survival of mammalian cells, whereas overexpression of antioxidant enzymes or application of RNS scavengers inhibits proliferation and cell survival.

Mitochondria, peroxisomes, and the xenobiotic metabolism by the cytochromes P450 may account for metabolic generation of O_2^-/H_2O_2. However, it is now clear that an important physiologic source of ROS in signal-transduction events is the

enzymatic complex NADPH oxidase. As mentioned before, the complex was first identified in phagocytes. The mechanism of activation of phagocytic NADPH oxidase, also known as NOX2, has been extensively studied and is a useful model for understanding the nonphagocytic isoforms of the oxidase. It is well established that activation of the oxidase involves the phosphorylation of the cytoplasmic subunits p47^{phox} and p67^{phox}, followed by the translocation of p47 ^{phox}, p67 ^{phox}, and p40 ^{phox} from the cytoplasm to the plasma membrane. In the plasma, these regulatory subunits interact with a complex that consists of gp91^{phox} and p22^{phox}, also known as cytochrome b_{558}. Activation of the complex also involves guanine nucleotide exchange on Rac2, and the metabolism of phospholipids (39, 102, 103, 104, 188). It was demonstrated that the small G protein Rac2 was essential for NOX2 activity. In cell-free systems, NOX2 activity is possible only if purified Rac2 is added to the assay system (202). In addition, Rac2$^{-/-}$ murine neutrophils were unable to produce O_2^- on incubation with various stimuli (195, reviewed in 97).

Phosphorylation of the cytoplasmic and regulatory component p47^{phox} causes conformational changes that disrupt an inhibitory intramolecular interaction between internal SH3 domains and a carboxy-terminal Pro-X-X-Pro motif. In this new conformation, the SH3 domains in p47^{phox} are exposed for binding to Pro-X-X-Pro motifs present in other components of NOX2 (19). In contrast to Rac2, p47^{phox} is not essential for NOX2 activity in cell-free systems, functioning primarily in the intact cell. P47^{phox} performs two major roles in the activation of NOX2: it acts as an adapter protein to facilitate binding of p67^{phox} with cytochrome b_{558} and regulates the NOX2 assembly induced by external stimuli (116).

Sundaresan *et al*. (354) described the elevation of the intracellular levels of H_2O_2 on stimulation of rat vascular smooth muscle cells with PDGF. Overexpression of catalase blocked the growth factor–stimulated increase of H_2O_2 and inhibited protein tyrosine phosphorylation (354). These findings were followed by the observations on the generation of H_2O_2 in fibroblasts stimulated with EGF (22). Since then, it became clear that a great variety of stimuli, including growth factors, cytokines, integrins, vasoactive modulators (*e.g*., bradykinin), and biomechanical forces (*e.g*., shear stress), stimulate mammalian cells to release ROS and RNS for signaling purposes (160).

To explain these phenomena, a search began for nonphagocyte NOX or gp91^{phox} homologues. The presence of p22^{phox}, a well-known component of the oxidase complex, in a great variety of cell types examined, led to a later description of seven different gp91^{phox} isoforms (38, 214). NOX1, NOX2, NOX3, and NOX4 are structurally similar (Fig. 13). The NOX5 isoform displays an amino-terminal cytoplasmic domain that contains a calmodulin-like domain with four calcium-binding sites. The other two isoforms, DUOX1 and DUOX2, have the same domain structural features as NOX5 and an additional membrane-spanning helix at the amino-terminal region that is homologous to heme peroxidases. O_2^- is the primary product of the five isoforms of the oxidase; however, DUOX1 and DUOX2 are isoforms of the complex capable of generating H_2O_2 as a primary product (7) (see Fig. 13).

Although a great deal of knowledge on the mechanistic aspects of the activation of NOX2 has been accumulated over the years, the mechanism of activation of the other isoforms is not completely understood. A major question remains unanswered: How does the binding of a growth factor to its cognate RPTK connect to the NOX isoforms, resulting in the production of ROS and protein tyrosine phosphorylation?

To deal with this problem, we examine some of the recent attempts to solve it.

To characterize the mechanism of H_2O_2 production on stimulation with PDGF in nonphagocytic cells, Bae *et al*. (23) used human hepatoma HepG2 cells ectopically expressing wild-type and PDGFR mutants. In wild-type PDGFR expressing HepG2 cells, stimulation with PDGF resulted in H_2O_2 production. However, in mutants displaying a replacement of Tyr740 and Tyr751 with Phe, recruitment and activation of PI3-K on stimulation with PDGF were prevented. In addition, PDGF stimulation of these mutants resulted in inhibition of H_2O_2 production.

The assembly of the signaling cascade responsible for ROS generation on growth-factor stimulation was further investigated in smooth muscle cells (SMCs) stimulated with PDGF (211). As mentioned earlier (354), H_2O_2 is produced in SMCs on binding of PDGF to its cognate receptor. In addition, crosstalk between different signaling systems permits the integration of the great variety of stimuli that a cell may receive under physiologic conditions. The transactivation of PDGFR on stimulation of G protein–coupled receptors (GPCR) resulted in a rapid increase in the ROS production, dependent on p22^{phox}. The participation of GPCR was evidenced by the use of *Pertussis toxin*, a powerful inhibitor of the G_i protein, and by the use of a neutralizing antibody against Gαi1, 2. Heterologous coupling of the PDGFR with the Gαi1, 2 subunit of the trimeric G protein, associated with the activation of p22^{phox} on NOX, defines a novel mechanism by which tyrosine phosphorylation and ROS production occur simultaneously.

A different cross-communication between PDGFR and NOX signaling pathways was observed in NIH3T3 fibroblasts (56). In these cells, redox regulation of the PDGFR signaling pathway involves the activation of the kinases, PKC and PI3K, which activate NOX with generation of H_2O_2. The authors suggested that these events be connected to the activation of Src kinase as a necessary condition for full activation of the PDGFR and production of H_2O_2.

Some similarities were found among the constituent elements of the signaling cascades triggered by different RPTKs. The signaling elements associated with the production of ROS by the EGFR signaling pathway were determined in rat SMCs. Cell stimulation with prostaglandin F2α enhances the phosphorylation of EGFR and the phosphorylation of the ERK1/2 MAP kinases and of Akt. Assembly of this signaling cascade leads to the induction of the expression of gp91^{phox}, the catalytic subunit of NOX, and production of O_2^- (110). Good experimental evidence exists for coupling EGFR and the signaling pathway activated by endothelin-1. Endothelin-1 is implicated in fibroblast proliferation that results in cardiac fibrosis. Transactivation of the EGFR and generation of ROS are critical steps in endothelin-1–mediated signaling events. Stimulated EGFR by endothelin-1 was found associated with the PTP SHP-2. SHP-2 activity was transiently inhibited during stimulation of rat cardiac fibroblasts with endothelin-1, and inhibition is prevented by NAC and diphenilene iodonium (60). The authors suggested that endothelin-1–mediated ROS production is asso-

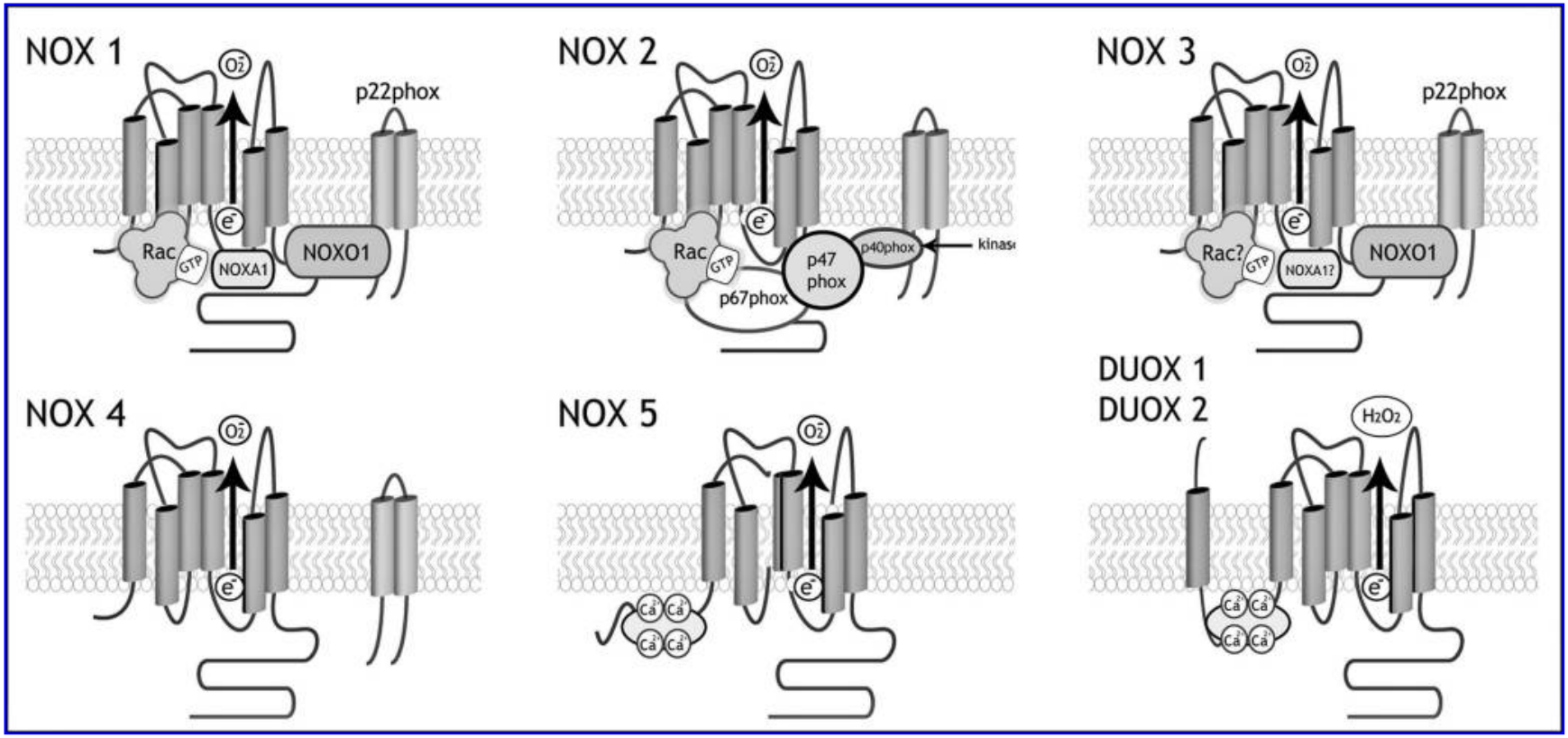

FIG. 13. Human isoforms of the NADPH oxidase complex. NOX1, NOX2, NOX3, and NOX4 are structurally similar among themselves. The four isoforms feature a C-terminal cytoplasmic domain that binds FAD and NADPH. The NOX5 isoform displays an N-terminal cytoplastic domain that contains a calmodulin-like domain with four calcium-binding sites. The other two isoforms, DUOX1 and DUOX2, have the same domain structural features as NOX5 and an additional membrane spanning helix at the amino-terminal region that is homologous to heme peroxidases. O_2^- is the primary product of the five isoforms of the oxidase. However, DUOX1 and DUOX2 are isoforms of the complex capable of generating H_2O_2 as a primary product.

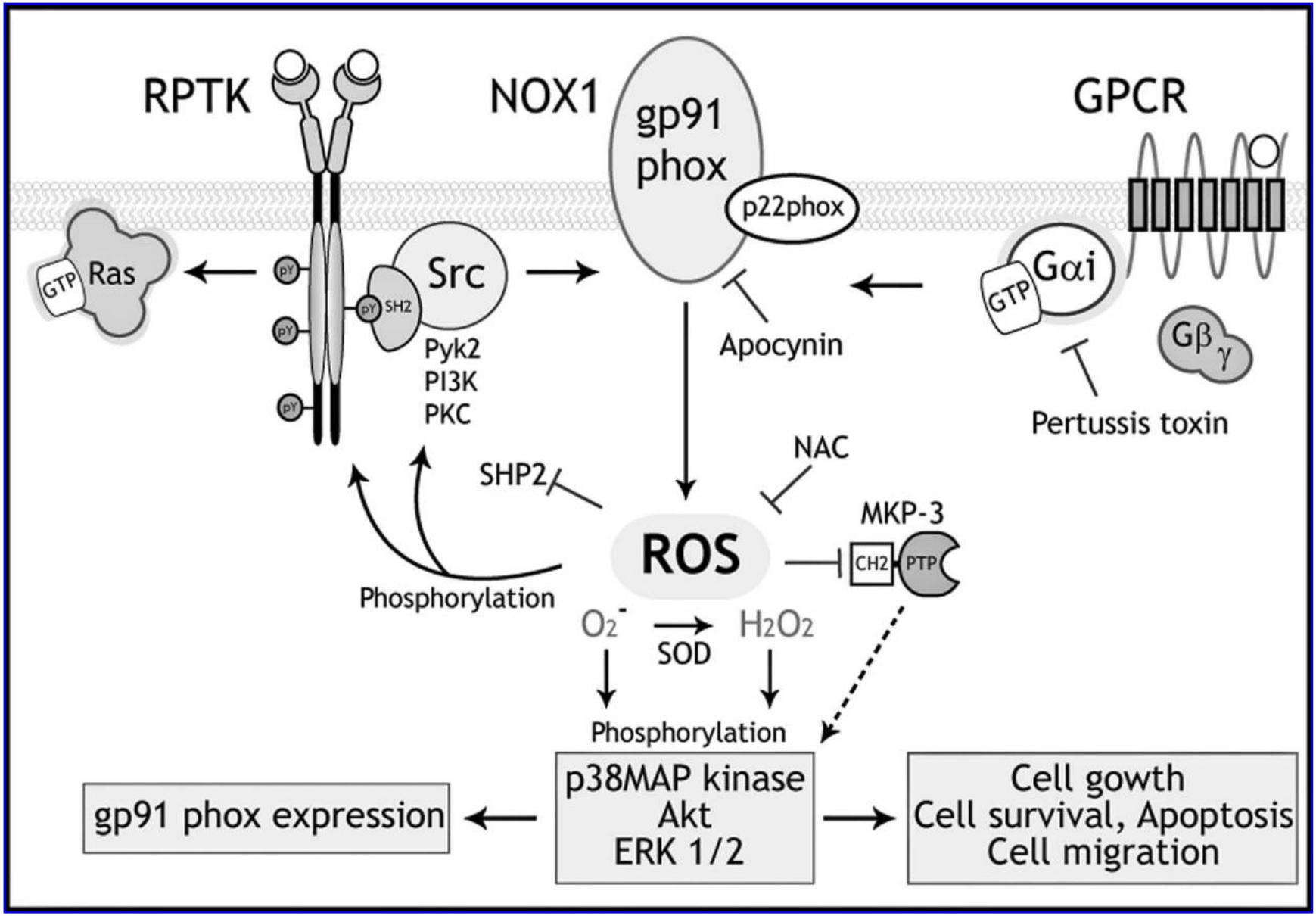
RPTK
NOX1
gp91
phox
p22phox
GPCR
GTP
Ras
pY
SH2
Src
Pyk2
PI3K
PKC
Apocynin
GTP
Gαi
Gβγ
Pertussis toxin
SHP2
NAC
MKP-3
ROS
CH2
PTP
Phosphorylation
O2-
SOD
H2O2
Phosphorylation
gp91 phox expression
p38MAP kinase
Akt
ERK 1/2
Cell gowth
Cell survival, Apoptosis
Cell migration

FIG. 14.

ciated with the oxidant-mediated transient inhibition of SHP-2 and shedding of heparin-binding EGF (HB-EGF), an EGF precursor associated with heparan sulfate at the extracellular matrix. These EGF-like ligands synthesized as transmembrane precursors are converted to the mature form by proteolytic cleavage (247). Stimulation of EGFR by endothelin-1 promotes the coupling between the RPTK and GPCR with release of HB-EGF (60).

Activation of the insulin-like growth factor type-1 (IGF-1) receptor in vascular SMCs results in H_2O_2-stimulated phosphorylation of Akt. Furthermore, signaling through the IGF-1 receptor stimulates tyrosine phosphorylation of Src kinase and Pyk2, which follows H_2O_2-stimulated phosphorylation of Ser 473 on Akt (17).

Early observations showed that human fat cells produced low levels of ROS on stimulation with insulin (212). Furthermore, oxidants such as vanadate, H_2O_2, pervanadate, and thiol-oxidizing compounds were described as insulinomimetic agents capable of stimulation of transport and utilization of glucose (79, 80, 151, 300). In addition to their insulinomimetic capacities, these compounds promote tyrosine phosphorylation of the IR β-chain (151). Further observations by Schmid *et al*. (328) provided evidence on the enhancement of IR autophosphorylation and tyrosine kinase activity by direct oxidation of Cys residues on the IR kinase domain. A specific redox-sensitive Cys residue in the IR kinase domain was found to be targeted by H_2O_2 in experiments performed with purified recombinant IR kinase domain fragments. Autophosphorylation of the IR is stimulated by H_2O_2 and required direct interaction of the oxidant with the kinase domain. Stimulation of phosphorylation was not detected in IR mutants in which the Cys1245 and Cys1308 residues were replaced by Ala residues (329).

Granulocyte colony-stimulating factor (G-CSF) and its cognate receptor play an essential role as hematopoietic growth factors for generation of granulocytes (233). Recently, it was described the induction of ROS by G-CSF in murine neutrophils. As mentioned earlier, NOX is a major generator of ROS in hematopoietic cells and G-CSF–induced ROS production starts with Ser phosphorylation of $p47^{phox}$ stimulated by Akt. The authors also demonstrated the participation of the Src family PTK Lyn on G-CSF induction of ROS. Inhibition of Lyn abrogated Akt phosphorylation and ROS production positioning Lyn upstream of NOX. Based on their findings, the authors suggested a "positive feed-forward loop" by which ROS stimulates Lyn activity, which in turn activates PI3K, Akt, and, in sequence, NOX, generating additional ROS (395).

Although the evidence presented in this section emphasize the participation of ROS generated by NOX isoforms in signal transduction, the mechanism by which RPTKs and other PTKs activate NOX and regulate ROS production is not completely understood. Compartmentalization of the NOX isoforms is one important aspect of this regulatory mechanism. The original observation by Hilenski *et al*. (162) that NOX1 and NOX4 have specific subcellular localization in SMCs provided important insights into the role of these oxidases in signaling processes.

Caveolins are a family of proteins with molecular mass ranging from 21 to 24 kDa, with three mammalian isoforms identified. Among them, caveolin-1 is widely expressed and serves as a scaffold protein involved in the recruitment and association of specific signaling complexes into caveolae (282). Caveolae and lipid rafts are cholesterol- and sphingolipid-rich microdomains of the plasma membrane where RPTKs, GPCRs, the Src family of PTKs, PKC, and small G proteins are localized (72). Recent observations by Vilhardt and van Deurs (376) account for the assembling of NOX in lipid rafts in endothelial cells stimulated by TNF-α, endostatin, or the Fas ligand. In SMCs, the presence of NOX1 in caveolae is thought to be involved in growth-promoting actions of the angiotensin type-1 receptor, a GPCR receptor (162). Activation of the angiotensin type-1 receptor involves its translocation to caveolae where the activation of NOX1 will occur. This activation promotes signaling events associated with Src family of PTKs, PKC, the small G protein Rac, and the EGFR (373).

The data discussed throughout this section revealed a pattern for signaling cascades that connect growth-factor receptors to the NOX isoforms. Src and Src family PTKs, PI3K, and Akt are major players in those signaling cascades by allowing the transmission and amplification of the signal. A simplified scheme is presented in Fig. 14.

B. Cell adhesion and the generation of reactive oxygen species

In the preceding section, we discussed tyrosine phosphorylation and redox signaling from the perspective of the interactions between growth factors and their cognate receptors. However, in multicellular organisms, growth-factor availability is not the only factor that governs cell growth and proliferation; positive and negative regulators delivered by the extracellular matrix and neighboring cells also play a role. Cell–cell contact-derived growth inhibition is an important mechanism of maintenance of growth homeostasis. The loss of cell–cell contact inhibition is observed in transformed cells that maintain the capacity to respond to mitogenic stimulation and proliferate beyond the monolayer restraints. Normal confluent cells grow in monolayers and have decreased levels of protein phosphotyrosine levels in association with the high activity of PTPs (27, 345). In addition, a negative control of cell growth by cell-cell contact was proposed, based on the reduced production of endogenous ROS and on the impairment of redox signaling mediated by growth-factor receptors (286). The author's assumption was based on the finding of reduced Rac-1 activity in confluent cell monolayers. As mentioned before, Rac-1 is an important component of the enzymatic complex NADPH oxi-

FIG. 14. Regular signaling cascades may connect RPTKs, GPCR, and the NOX isoforms. Activation of RPTKs and/or GPCR connects to the activation of NOX isoforms through Tyr/Ser/Thr phosphorylation of the oxidase regulatory components. Activation of NOX leads to the production of ROS. Elevated intracellular levels of ROS result in enhanced levels of tyrosine phosphorylated proteins because of inhibition of PTPs. Enhanced phosphorylation levels activate the MAP kinases, Akt, and promote the expression of gp91phox. Reducing agents, such as *N*-acetylcysteine, NOX inhibitors, RPTK inhibitors, and GPCR inhibitors, modulate these signaling cascades.

dase, absolutely necessary for the production of ROS involved in mitogenic signaling by growth-factor receptors. Furthermore, the activity of Rac-1 is required for cell-cycle progression in the G_1–S-phase boundary of the cell cycle (267). However, different from Rac-1 and Rap-1, which have their GTPase activity negatively regulated in confluent cells, p21Ras is unaffected by cell density (168, 286, 313).

Conversely, Rac-1 inhibition in high-density cell cultures could be mediated by oxidants produced after integrin engagement (382). It has been reported that integrin engagement in addition to growth factor–mediated signaling involves the production of ROS. Rac-1 stimulated mitochondrial production of ROS after integrin activation, NF-κB activation, and changes in cell shape (382). Later reports described that after contacts between the extracellular matrix and the integrins, a transient flux of H_2O_2 occurred. Hydrogen peroxide produced under these conditions came essentially as a byproduct of 5-lipoxygenase, with a smaller contribution of NOX isoforms (62). In a recent report, it was shown that both mitochondria and 5-lipoxygenase are involved in integrin-mediated production of ROS. Mitochondria-derived ROS production is restricted to the early phase of cell adhesion, whereas 5-lipoxygenase is responsible for the late generation of the oxidants associated with cytoskeleton remodeling and actin stress fiber formation on cell spreading (357).

ROS production associated with adhesion processes promotes protein tyrosine phosphorylation. In addition to RPTKs, cytoplasmic PTKs associated with adhesion-dependent cell growth can also be redox regulated. Early reports pointed to a redox-based regulation of the phosphorylation levels of Src and members of the Src family of kinases in T lymphocytes and NIH 3T3 murine fibroblasts (272, 302). Later, we and others demonstrated the stimulation of tyrosine phosphorylation on Src kinase after exposure of NIH 3T3 cells to ·NO/RNS–generating compounds (4, 263). More important, ·NO-mediated activation of Src kinase did not depend on the previous dephosphorylation of its carboxy-terminal p-Tyr527 residue (78). Further evidence on redox regulation of Src on integrin en-

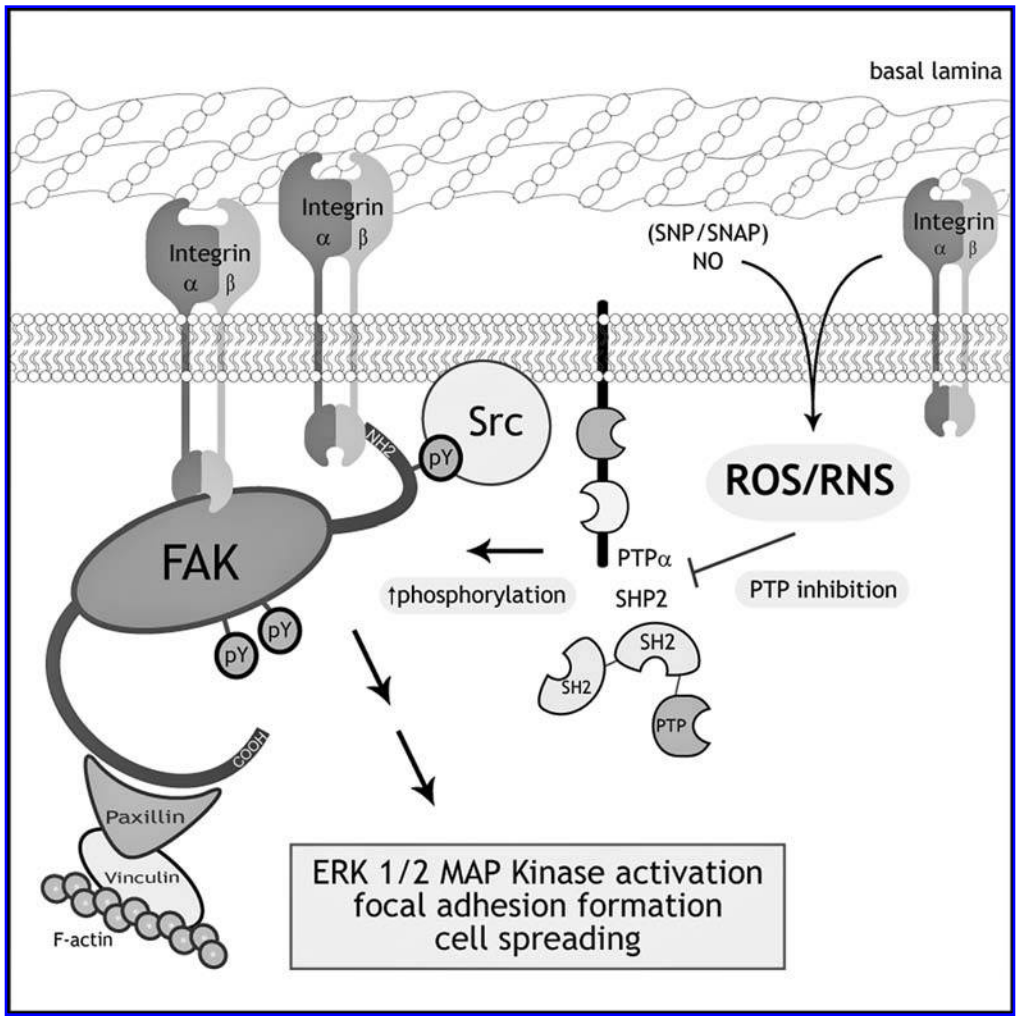

FIG. 15. Oxidant-mediated adhesion signaling. ROS and RNS can inhibit receptor and non-receptor TPTs stimulating tyrosine phosphorylation-dependent downstream adhesion-related signaling events. Src kinase, a major component of focal adhesions, is also subjected to redox regulation. Redox signaling through Src leads to activation of the EKR1/2 MAP kinases, focal adhesion complex formation, and cell spreading.

gagement came from studies with murine fibroblasts that were submitted to detachment and readhesion to the extracellular matrix. The authors showed that after integrin ligation, Src is oxidized and activated. Kinase oxidation and activation were inhibited in cells pretreated with *nor*-dihydroguairetic acid, an inhibitor of 5-lipoxygenase (128).

Recent structural studies suggest a novel redox-based regulatory mechanism for all members of the Src family of kinases. The authors described a unique disulfide bond located in the SH2 domain of Csk, the cytoplasmic PTK that downregulates Src activity, as mentioned earlier. Formation of this disulfide bond in the SH2 domain of Csk decreases the enzyme activity by an order of magnitude. The authors concluded, after normal mode analysis and molecular dynamics calculations, that disulfide bond formation exerts its effects on residues within the kinase domain. They concluded with the assumption that Csk activity may be redox regulated after a reversible cross-linking of Cys122 and Cys164 in the SH2 domain of the enzyme. This may have an impact on the activities of Src kinase and other members of the Src family in cells under oxidative-stress conditions (257).

Downstream from Src at focal adhesion sites, we find FAK that has its tyrosine phosphorylation levels stimulated after exposure of NIH3T3 murine fibroblasts to ·NO/RNS-generating compounds. In our studies, we found low basal levels of Src kinase that was co-immunoprecipitated with FAK in nonstimulated murine fibroblasts. After exposure of cells to ·NO donors, higher levels of Src associated with FAK were observed. Our findings suggested that ·NO promotes the formation of the Src–FAK complex with stimulation of tyrosine phosphorylation levels in both kinases. Alterations on the tyrosine phosphorylation levels of FAK promoted by ·NO interfere with the *de novo* formation of focal adhesion (124). These findings were corroborated in endothelial cells, where cell adhesion and spreading, and formation of focal adhesions were inhibited by ·NO. Further, treatment of endothelial cells with ·NO donors reversibly released tractional forces exerted by focal adhesions and stress fibers (133).

Alternatively, inhibition of PTPs by ·NO was suggested as a cause for the increased tyrosine phosphorylation levels of FAK after •NO stimulation (263). Recent findings on redox regulation of FAK after integrin engagement gave support to these observations. It was reported that integrin-induced ROS production is required to oxidize and inhibit the activities of low-molecular-weight PTP and SHP2. Inhibition of both PTPs causes upregulation of the phosphotyrosine levels on FAK. Conversely, inhibition of integrin-mediated ROS production prevents FAK phosphorylation and downstream signaling events, such as the activation of ERK1/2 MAP kinases, focal adhesion formation, and cell spreading (62, 357) (Fig. 15).

Oxidative-stress conditions may also regulate FAK activity in cells undergoing apoptosis. Sonoda *et al.* (344) found, in human glioblastoma cells exposed to H_2O_2, a rapid induction of tyrosine phosphorylation levels of FAK followed by the decrease of phosphorylation concomitant with apoptosis. Serine phosphorylation on Akt was observed on treatment of glioblastoma cells with H_2O_2. Based on these studies, the authors proposed an antiapoptotic role for FAK in cooperation with PI3K and Akt in H_2O_2-induced apoptosis in glioblastoma cells (344).

VII. REDOX SIGNALING BY SMALL G PROTEINS

A. Redox modulation and production of reactive oxygen species by small G proteins

Earlier in this review article, we discussed the participation of the small G protein Rac in the generation of ROS by phagocytic and nonphagocytic cells. In phagocytes, Rac is implicated in the function of NOX2 (20). However, the production of ROS by other cell types stimulated by growth factors or cytokines in addition to Rac, includes the participation of p21Ras (355). This study demonstrated that cells expressing constitutively active mutants of both small G proteins produce high levels of ROS. The authors also provided experimental evidence suggesting that Rac is positioned downstream with respect to p21Ras. Expression of a dominant-negative mutant of Rac inhibited p21Ras-stimulated ROS production (355). Besides ROS generation, Rac was shown to be required for cell proliferation and progression through the G_2/M phases of the cell cycle (267).

Studies with NIH3T3 fibroblasts stably transformed with a constitutively active isoform of p21Ras, V12Ras, showed a large production of O_2^- associated with a high rate of proliferation. Transfection of NIH3T3 V12Ras fibroblasts with dominant-negative mutants of Rac and p21Ras, or treatment of the cells with a p21Ras inhibitor or with a NOX inhibitor, suppressed O_2^- generation. In addition, the use of NAC inhibited the mitogenic activity of the V12Ras-transformed fibroblasts (174). These findings suggest that although p21Ras and Rac contributed positively to ROS production, they may also serve as targets for redox modulation.

Small G protein–mediated production of ROS may also result in replicative senescence. Human diploid fibroblasts expressing V12Ras produce high intracellular levels of ROS, particularly in mitochondria, and undergo growth arrest (174, 222). V12Ras-mediated growth arrest and senescence was partially inhibited by coexpression of an activated form of Rac. Inhibition of production of ROS by placing the cells in a 1% O_2 environment resulted in reversion of growth arrest and senescence (222). One aspect of the participation of small G proteins in the intracellular production of ROS should be emphasized, in most cases in which overexpression of the activated forms of these signaling proteins stimulates ROS production; inhibition of NOX prevented ROS production (174, 267). Thus, activated forms of p21Ras and Rac might promote the assembly of the NOX isoforms, creating the proper conditions for ROS generation. Nevertheless, a very important question remains: are signals derived from activated small G proteins sufficient to generate ROS without the participation of RPTKs?

In normal cells, growth-factor stimulation of RPTKs leads to recruitment and activation of p21Ras (see Fig. 6), which will activate Rac with assembling of NOX components in the cytoplasm. Conversely, cancer cells produce constitutively higher levels of ROS and other oxidants than do normal cells (171). It appears that in this case, ROS production can proceed without the participation of RPTKs. The activated forms of p21Ras and Rac stimulate ROS generation by promoting the assembly and activation of NOX isoforms or stimulate mitochondrial production of these reactive species, or both (95, 222, 362).

As mentioned earlier, signaling proteins such as RPTKs like the EGFR and PDGFR, cytoplasmic PTKs like Src and FAK, and small G proteins like Rac and p21Ras may contribute to ROS production. In addition, they serve as targets for redox regulation. This was particularly explored in depth for the small G protein p21Ras. In 1995, Lander and co-workers (218, 219) identified p21Ras as a critical target for many redox modulators, including ·NO, H_2O_2, and thiol oxidants. *In vitro* studies using recombinant p21Ras and ·NO donors in the presence of O_2 demonstrate that nitrogen dioxide radical ($\cdot NO_2$) induced a conformational change in p21Ras associated with GDP/GTP exchange. The same group extended their observations, demonstrating unequivocally that p21Ras was the target for redox regulation. Besides the ·NO donors, oxidants such as H_2O_2, hemin, and Hg^{2+} were also capable of stimulating the activity of p21Ras. Structural studies demonstrated that Cys118 that resides on a loop close to the guanine nucleotide–binding pocket could be redox regulated. Redox modifications of the residue appears to promote guanine nucleotide exchange, and replacement of Cys118 by Ser (C118S) results in inhibition of signaling events downstream with respect to p21Ras (216). The nature of the oxidizing agent appears to determine the redox modification on Cys118 and its effects on p21Ras activity. The nature of the oxidative modifications on p21Ras in NIH3T3 cells expressing either the wild-type isoform of H-Ras or the C118S mutant varied if the cells were exposed to diamide, H_2O_2, or to ·NO donors (241). Structural, mutational, and chemical studies showed that four residues [118, 181, 184, and 186] among the total six Cys residues on H-Ras are surface exposed (146, 216, 268). Low-molecular-weight and protein mixed disulfides of H-Ras are formed on incubation of NIH3T3 fibroblasts with diamide. Cys186 is apparently the cysteine residue that is predominantly modified under these conditions. Moreover, *S*-nitrosocysteine, a transnitrosating compound used in millimolar concentrations, preferentially induces S-nitrosation at Cys118 on H-Ras (241). Because the concentrations of the transnitrosating reagent used in the study were out of physiologic range, the other three cysteine residues, Cys181, 184, and 186, were also nitrosated (241). Considering the situations in which cells are exposed to a physiologic flux of ·NO, redox-mediated alterations on p21Ras might occur essentially through the redox-active Cys118. Cys118 is located in the highly conserved nucleotide-binding motif Asn-Lys-Cys-Asp, present in all isoforms of p21Ras and other p21Ras-related small G proteins (154, 156). Inside the motif, the thiol of Cys118 is located between basic and acidic side chains, a condition required for S-nitrosation (160). Thus, it is expected that S-nitrosation of Cys118 will promote guanine nucleotide exchange. However, recent studies by the Campbell group (157, 155) demonstrated that S-nitrosated p21ras (p21Ras-SNO) does not directly promote p21Ras guanine nucleotide exchange. In their studies, the authors used wild-type recombinant p21Ras treated with ·NO and O_2 (a condition that generates $\cdot NO_2$). A nucleotide adduct characterized as 5-guanidino-4-nitroimidazole diphosphate was released under these conditions, and it was not detected in C118S mutants of p21Ras submitted to the same treatment. These findings suggest that p21Ras Cys 118 thiol plays a major role in the formation of the nucleotide adduct and consequent release of p21Ras-bound GDP. Formation of p21Ras-SNO was observed only after release of p21Ras-bound GDP. In addition, the authors suggested that formation of p21Ras-SNO might prevent further reaction with $\cdot NO_2$. If denitrosation of p21Ras-SNO, however, occurs by transfer of •NO to GSH, Trx, or other cellular thiol-containing molecules, denitrosated p21Ras can react again with $\cdot NO_2$, and a new cycle of guanine nucleotide exchange may begin. Thus, one can speculate that although S-nitrosation of Cys118 is not directly responsible for p21Ras GDP/GTP cycling, the absence of this posttranslational modification on p21Ras prevents guanine nucleotide exchange.

In addition to ·NO, other ROS and RNS, including O_2^-, $H_2O_2/OH\cdot$, and $ONOO^-$, have been implicated in the p21Ras-mediated signaling pathways. With the exception of H_2O_2, which is not capable of facilitating p21Ras guanine nucleotide release, the other ROS appears to stimulate nucleotide release, apparently following a mechanism similar to that described for $\cdot NO/O_2$. Thus, biologic antioxidants and/or reductants such as ascorbate and GSH will be critical in redox-mediated activation of p21Ras (155, 156).

B. Redox modulation of p21Ras and induction of protein phosphorylation

As mentioned earlier, the GTP-associated form of p21Ras interacts with effector proteins, which are signaling proteins with a diversity of functions (377). Early observations accounted for the identification of PI3K as one of the effectors recruited by ·NO-activated p21Ras. Downstream to PI3K, the protein kinase Akt and members of MAP kinase family were found to propagate this ·NO-mediated signaling event (90). The same group characterized other effectors recruited by •NO-activated p21Ras. One of these effectors, the protein Ser/Thr kinase Raf-1, interacted transiently with p21Ras on exposure of Jurkat T cells to an RNS source. The authors used Jurkat T cells expressing the C118S mutant of p21Ras exposed to an RNS source and were unable to co-immunoprecipitate p21Ras and Raf-1. These results strongly suggested that Raf-1 is part of a signaling cascade triggered by the interaction of NO/RNS with the Cys118 residue of p21Ras (89). Activation of the MAP kinases family, including the ERK1/2, JNKs, and p38, was also observed as a result from RNS-mediated activation of p21Ras (217).

Our group took a further step regarding the connections between the ·NO-activated p21Ras-ERK1/2 MAP kinases signaling cascade and the stimulation of tyrosine phosphorylation by the EGFR-signaling pathway. In our early studies, we described in murine fibroblasts the ·NO-mediated stimulation of tyrosine phosphorylation of a set of proteins that were part of the EGFR-signaling pathway (293). Later, we found that a similar set of proteins in rabbit aortic endothelial cells was phosphorylated on tyrosine on incubation of the cells with different sources of ·NO/RNS. Stimulation of p21Ras was also observed under these conditions. Inhibition of p21Ras activity with specific pharmacologic inhibitors or with overexpression of its negative-dominant form (N17Ras) resulted in inhibition of the ·NO/RNS-stimulated tyrosine phosphorylation. Downstream to p21Ras, stimulation of the ERK 1/2 MAP kinases was also abolished under these conditions. Upstream to p21Ras, ERK1/2 MAP kinases target the EGF receptor. Incubation of rabbit endothelial cells with ·NO donors resulted in activation of the EGF-receptor autophosphorylation. PD98059, a pharmacologic

inhibitor of the protein kinase MEK and consequently an indirect inhibitor of the ERK1/2 MAP kinases, inhibited this activation. In addition, the EGFR autophosphorylation was not responsive to RNS/·NO stimulation in N17Ras-expressing endothelial cells. We concluded that the p21Ras-initiated signaling pathway is connected to ·NO-stimulated overall tyrosine phosphorylation and also involved the ERK1/2-mediated transactivation of the EGFR (309). Recently, we also described that the RNS/·NO-mediated activation of the ERK1/2 MAP kinases may as well stimulate one of their downstream nuclear substrates, the transcription factor Elk 1. Activation of Elk 1 was followed by induction of specific cyclins and cyclin-dependent kinases, resulting in cell-cycle progression (Rocha Oliveira *et al*., unpublished observations) (Fig. 16).

Stimulation of Elk 1 by RNS and ROS was also observed in Jurkat T lymphocytes. By using specific inhibitors of elements of the signaling pathway, the authors were able to demonstrate that S-nitrosation of Cys118 on p21Ras, its effector PI3K, and the ERK1/2 MAP kinases was redox-activated upstream to Elk 1 (89).

Production of ·NO through stimulation of neuronal cells with the neurotrophin nerve growth factor resulted in the activation of the p21Ras–ERK1/2 MAP kinases survival-signaling pathway and nuclear translocation of Trx (24). In addition, under

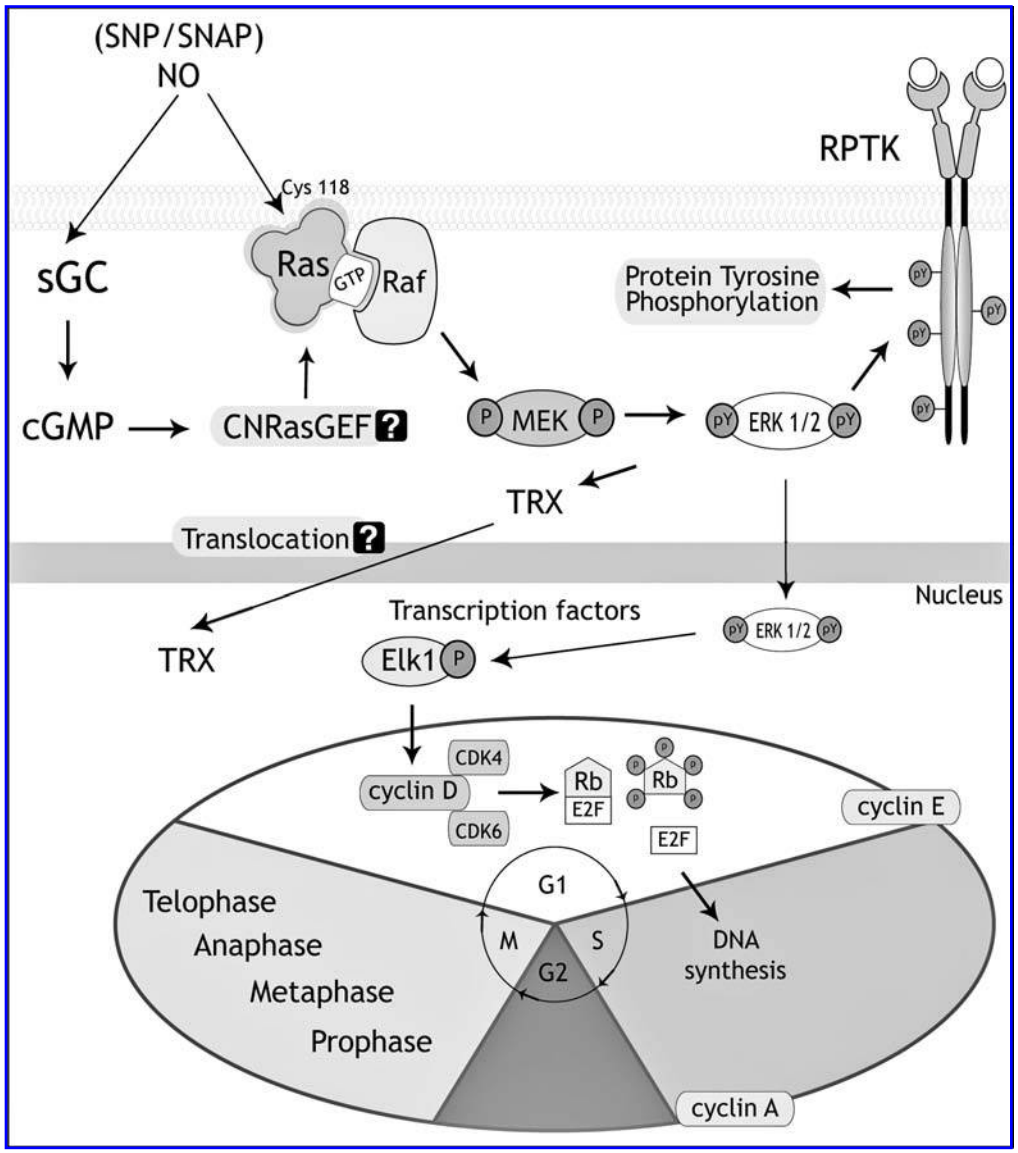

FIG. 16. Ras signaling pathways are stimulated by exogenous and endogenous sources of nitric oxide (·NO). ·NO triggers multiple signaling pathways through *S*-nitrosation and/or cGMP, *S*-nitrosation and/or cGM P guanine nucleotide exchange factors stimulate p21Ras. Activation of p21Ras leads to recruitment and activation of the Raf/MEK/ERK1/2 signaling cascade with increased cell proliferation. Elevated concentrations of ·NO donors and high levels of ·NO produced by the inducible NOS trigger the Ras survival-signaling pathway with the participation of the redox regulator Trx.

conditions of nitrosative stress, ·NO was shown to stimulate the same survival-signaling pathway. Nuclear translocation of Trx associated with cell survival was observed in HeLa cells exposed to a nitrosating agent. Accordingly, inhibition of p21Ras or inhibition of tyrosine phosphorylation and activation of the ERK1/2 MAP kinases resulted in inhibition of ·NO-induced Trx nuclear translocation and decreasing cell viability (11) (see Fig. 16).

Altogether, these observations and others described in the preceding sections of this review article led us to conclude that reversible protein oxidation, specifically Cys oxidation, is in synergy with protein phosphorylation. However, a relation between tyrosine phosphorylation and other oxidative posttranslational modifications like protein tyrosine nitration still is a debatable issue. In an attempt to fill the gaps, let us explore, in the final section, the pro and con aspects related to signaling events mediated by protein tyrosine nitration.

VIII. TYROSINE NITRATION AND REDOX SIGNALING

A. Nitrating species: peroxynitrite, nitrogen dioxide, and derived species

Among the three isoforms of the enzyme NOS, the inducible isoform (iNOS) plays a major role in the mediation of host inflammatory reactions. During pathologic conditions associated with the inflammatory process, expression of the enzyme is strongly upregulated at the transcriptional level. Endotoxins, cytokines, and the combination of both are strong inducers of iNOS (203). On activation, the enzyme efficiently produces copious amounts of ·NO and its primary oxidation product nitrite (NO_2^-). NO_2^- was found to be metabolized *in vitro* by the important inflammatory mediators, hypochlorous acid or myeloperoxidase, to generate the nitrating species $\cdot NO_2$ (100, 374). Later observations by the same group demonstrated that activated human neutrophils convert NO_2^- into $\cdot NO_2$ and the chlorinating species nitryl chloride. Under these conditions, angiotensin-converting enzyme from endothelial cells was nitrated on tyrosine and became inactivated (101). Thus, activated neutrophils, through myeloperoxidase, efficiently nitrate tyrosine residues, and this process is part of a general oxidative inflammatory response.

In addition to the role of myeloperoxidase in nitration of tyrosine residues, the enzyme's primary function is to catalyze the reaction between H_2O_2 and chloride ions, producing hypochlorous acid (381). Hypochlorous acid was shown to be highly reactive with nonprotein and protein thiols (388), making the oxidant a potential redox regulator of PTP activities. Recently, hypochlorous acid was found to induce cellular tyrosine phosphorylation in both T and B lymphocytes, activate the ZAP-70 tyrosine kinase, and induce cellular calcium signaling in a tyrosine kinase–dependent manner. These signaling events also occurred in T-cell lines that did not express the T-cell receptor, indicating the ability of hypochlorous acid to bypass normal receptor control (323).

Another nitrating species is the strong oxidizing compound peroxynitrite ($ONOO^-$). At physiologic pH, $ONOO^-$ is converted to its protonated form, the peroxynitrous acid (ONOOH), a reactive species that rapidly undergoes homolytic cleavage to yield $\cdot NO_2$ and ·OH. Alternatively, ONOOH isomerizes to nitrate (NO_3^-). Thus, formation of the nitrating radical $\cdot NO_2$ from $ONOO^-$ is mostly regulated by reactions with thiols, by the isomerization rates, and by the reaction with CO_2. The reaction of $ONOO^-$ with CO_2 yields the nitrosoperoxocarbonate anion ($ONOOCO_2^-$), which is physiologically relevant, because CO_2 is ubiquitous, and its concentration increases during interruptions of tissue oxygen transport. $ONOOCO_2^-$ undergoes homolytic cleavage to yield the radicals $\cdot NO_2$ and $\cdot CO_3^-$. Alternatively, rearrangements yielding NO_3^- and CO_2 may occur as well (40).

Tyrosine nitration may be a result of an inflammatory response; $ONOO^-$-mediated tyrosine nitration has also been viewed as a marker for the extent of RNS production. Extensive protein tyrosine nitration leads to inactivation of proteins and is considered an indicator of cellular stress associated with pathologic conditions. Under these conditions, tyrosine-nitrated proteins are usually considered dead-end products that are inevitably destined for protein degradation (346). However, physiologic levels of protein tyrosine nitration have been detected (132, 204), raising the issue of whether it may also perform a role as a posttranslational modification with signaling functions (266). To perform signaling functions, tyrosine nitration may interfere (positively or negatively) with the existing tyrosine phosphorylation/dephosphorylation signaling pathways. Alternatively, it may perform its signaling functions on pathways based on tyrosine nitration/denitration.

B. Tyrosine nitration and protein phosphorylation

Protein tyrosine nitration may interfere directly or indirectly with tyrosine phosphorylation/dephosphorylation signaling pathways. Protein tyrosine nitration of signaling proteins associated with tyrosine phosphorylation/dephosphorylation signaling cascades was detected in mammalian cell cultures exposed to reagent $ONOO^-$. The levels of tyrosine phosphorylated proteins decreased, and protein tyrosine nitration increased after exposure of bovine pulmonary aortic endothelial cells to reagent $ONOO^-$ (134).

Specific components of different signaling cascades were found to be tyrosine nitrated. In the human epidermoid carcinoma cell line A431 that overexpressed EGFR, $ONOO^-$ promoted dimerization of the receptor, possibly through intermolecular dityrosine cross-linking. Downstream to the dimerized EGFR, tyrosine phosphorylation of PLCγ was significantly reduced (375).

As previously mentioned, activation of the antiapoptotic kinase Akt might result from RPTK activation. It was shown that high concentrations of $ONOO^-$ induce apoptosis and interfere with RPTK signaling through nitration of tyrosine residues that otherwise would be phosphorylated (375). However, at relatively low concentrations, $ONOO^-$ administered as a bolus addition to a preformed solution or generated by O_2^- and ·NO obtained from the hydrolysis of an $ONOO^-$ donor, 3-morpholinosydnoimine (SIN-1), promote the Akt survival-signaling pathway. Akt activation is rapid and followed by phosphorylation and activation of glycogen synthase kinase-3, a substrate for Akt. Inhibitors of PI3-K and of the PDGFR pre-

vented $ONOO^-$-mediated Akt activation, suggesting that $ONOO^-$-mediated Akt activation occurs as a survival-signaling response (201).

Protein kinases in the cytoplasm are also affected by the exposure of cells to reagent $ONOO^-$ or to SIN-1. PI3K was identified as a target for protein tyrosine nitration in lysates from RAW 264.7 macrophages incubated with 1 m*M* $ONOO^-$. The target was p85, the regulatory subunit of PI3K, which on activation associates with p110, the catalytic subunit of PI3K. Tyrosine nitration on p85 inhibits its association with p110 and consequently prevents the signals relayed by PI3K, thus opposing tyrosine phosphorylation by tyrosine nitration (153).

Tyrosine nitration of p130Cas a major component of focal adhesion complexes is predominant as compared with tyrosine phosphorylation in neuroblastoma cells exposed to a high flux of $ONOO^-$ generated from SIN-1 (315). Conditions like those described led to an erroneous conclusion that tyrosine nitration essentially prevents the regular cycle between phosphorylated and nonphosphorylated states of tyrosine residues. Such is a simplistic way to interpret the participation of tyrosine nitration on redox signaling.

Because $ONOO^-$ is a powerful thiol oxidant, inhibition of PTP activities would equal the effects of ROS and other RNS such as ·NO and nitrosothiols. Early *in vitro* observations accounted for the inactivation of three human recombinant PTPs. Concentrations of reagent $ONOO^-$ as low as 2 μM caused a rapid and efficient inactivation of the receptor T-cell tyrosine phosphatase (CD45), the leukocyte RPTP LAR, and the cytoplasmic tyrosine phosphatase PTP1B. Inactivation was caused by the $ONOO^-$-mediated oxidation of the essential thiolate anion present in all PTP active sites. In contrast to the inhibition caused by nitrosating agents such as GSNO, which was completely reversed after treatment with DTT, PTP inactivation promoted by $ONOO^-$ was irreversible (360). Based on these observations, the authors suggested that if $ONOO^-$ mediated inactivation of PTPs *in vivo*, this would result in a net increase in tyrosine phosphorylation.

Several studies, by using a variety of cell types, neuroblastoma cells, human T lymphocytes, and human erythrocytes, incubated with reagent $ONOO^-$, showed elevation in cellular nitrotyrosine and phosphotyrosine levels. Changes on tyrosine phosphorylation induced by $ONOO^-$ exhibited a concentration dependence on the oxidant. These changes in phosphotyrosine levels were transient and responsive to relatively low concentrations of $ONOO^-$ (50, 232, 242). In T lymphocytes, transient increase in tyrosine phosphorylation even after mild exposure to $ONOO^-$ may deplete GSH and other intracellular reducing agents. This form of mild oxidative stress potentially sensitizes intracellular PTPs to oxidative inactivation, with consequent increase in the intracellular levels of phosphotyrosine (262).

Studies using human erythrocytes incubated with reagent $ONOO^-$ at concentrations ranging from 10 to 100 μM showed stimulation of tyrosine phosphorylation levels of band 3 transmembrane protein. Increase in phosphotyrosine levels of the cytoplasmic domain of band 3 protein was accompanied by reversible inhibition of PTPs. The glucose metabolism of $ONOO^-$-treated erythrocytes was stimulated, and production of lactate was enhanced (242). $ONOO^-$, at levels ranging from 30 to 250 μM, stimulated tyrosine phosphorylation of the members of the Src family of PTK, lyn, hck, c-fgr, and fyn in human erythrocytes exposed to the oxidant. Higher concentrations of $ONOO^-$ inhibited the activity of all kinases studied (243). Increased levels of tyrosine phosphorylation of Src kinase were observed in bovine brain synaptosomes exposed to $ONOO^-$ (50–500 μM). At the millimolar level, $ONOO^-$ completely inhibits tyrosine phosphorylation, and tyrosine nitration becomes predominant, suggesting a biphasic effect of the oxidant on tyrosine kinases.

In addition to Src and the Src family of PTKs, other kinases had their activities regulated by $ONOO^-$. Rat liver epithelial cells exposed to a steady-state concentration of $ONOO^-$ (6.5 μM) for 3 min showed a transient dual phosphorylation of the three major MAP kinase pathways, ERK1/2, p38, and JNK. Levels of tyrosine phosphorylation were downregulated in the three kinases if cells were grown in medium supplemented with selenite, a cofactor for GSH peroxidase (322). Activation of ERK1/2 and p38 MAP kinases in PC12 cells, which endogenously express receptors for EGF and NGF, was observed after treatment with 500 μM $ONOO^-$. The authors demonstrated that $ONOO^-$ rapidly activated the two MAP kinases through the mediation of the receptors for EGF and NGF, calcium/calmodulin-dependent kinase II, and the Src-related PTKs (185).

These results indicated that like other oxidants, $ONOO^-$ can modulate tyrosine phosphorylation–mediated signaling cascades. The concentrations of $ONOO^-$ given to the cells will determine the occurrence of positive or negative modulation.

Manipulation of the concentrations of $ONOO^-$ by a bolus addition of preformed solutions of the oxidant to the cells in culture yielded important information about signaling associated with tyrosine nitration. This approach has its own pitfalls, because concentrations of $ONOO^-$ have arbitrarily been given to different cell types, which would respond differently. Therefore, the physiologic occurrence and significance of signaling by tyrosine nitration and its relation with tyrosine phosphorylation could not be fully understood.

The problems arising from the use of a bolus addition of aliquots of preformed $ONOO^-$ solutions to cultured cells were recently discussed (215). The author used specially developed Windows-based software to predict that bolus addition of $ONOO^-$ will not reflect the situation in biologic systems. Lancaster's predictions were based on the fact that in biologic conditions, $ONOO^-$ is formed from the extremely efficient reaction of ·NO with O_2^- (see earlier for the rate constant); each of them will be produced as a flux of varying rates, depending on the environment and conditions. Because of the extremely high efficiency of the reaction that produces $ONOO^-$ under most biologic conditions, an excess of either ·NO or O_2^- will be found. With increasing fluxes of one reactant (·NO) and a constant flux of the other (O_2^-), the formation of $ONOO^-$ will increase linearly with ·NO flux until the rate is equal to the O_2^- flux, and above this value, the concentration is constant. Therefore, $ONOO^-$ formation will be determined by the flux of the reactant produced at a slower rate (186).

The actions of $ONOO^-$ as oxidant or nitrating agent have also been investigated in several situations in which this reactive species is produced by a diffusion-controlled reaction (1.9×10^{10} M/sec) between ·NO and O_2^- (106, 132, 200, 393). Differences in the concentrations of both reactants would lead to different outcomes. For instance, the excess of ·NO may force

the reaction to produce nitrosating or nitrosylating species, whereas the excess of O_2^- would tend to produce strong oxidants such as $^{\bullet}OH$ (401). Results obtained in *in vitro* experiments showed that the balance between ·NO and O_2^- would determine nitrosative or oxidative stress conditions. In reactions in which an excess of ·NO is present, the formed $ONOO^-$/ONOOH would react with available ·NO, forming $\cdot NO_2 + NO_2^-$. $\cdot NO_2$ in turn would react with additional ·NO, producing N_2O_3, a potent nitrosating agent. This may shift the oxidative stress promoted by $ONOO^-$/ONOOH to a nitrosative stress promoted by N_2O_3. In this case, GSH may attenuate either oxidative or nitrosative stress by detoxifying the common mediator of both events: $ONOO^-$ (387). Thus, the presence of reducing systems and transition metals that potentially alter the levels of ·NO, O_2^-, and $ONOO^-$, would change the final products of the reaction. Under defined conditions for the reaction between ·NO and O_2^-, transition metals and reducing systems would promote the generation of distinct byproducts such as $^{\cdot}OH$, NO^+, NO^-/HNO, and $^{\cdot}NO_2$ (21, 82). Therefore, the simultaneous addition of exogenous sources of ·NO and O_2^- in biologic systems will not always reflect the actions of $ONOO^-$ in these systems.

Appropriate stimulation of signaling receptors, such as growth factor receptors, hormone receptors, and mechanotransducers for shear stress in endothelial cells, activates the cellular sources of ROS and RNS. *In vivo* generation of $ONOO^-$ is likely to occur from the reaction of different fluxes of NO and O_2^-, resulting from different types of cell stimulation. Potential endogenous sources of NO and O_2^- include the mitochondria and the enzymes xanthine oxidase, NOX, and NOS.

From this perspective, several experimental models were examined: Interleukin 1β (IL-1β) was shown to stimulate a rapid concentration-dependent increase in O_2^- in rat pulmonary microvascular smooth muscle cells. Upregulation of iNOS and production of iNOS-derived ·NO yielded $ONOO^-$ in this system (41). Similar findings were reported in rat pleural mesothelial cells exposed to asbestos and IL-1β (65).

Peripheral blood monocytes were stimulated with immobilized anti-CD3 monoclonal antibodies, and increasing tyrosine nitration of a group of proteins was observed. Preincubation of the cells with increasing concentrations of an NOS inhibitor and a SOD mimetic progressively inhibited overall tyrosine nitration (50).

Protein tyrosine nitration caused by endogenous processes was detected in isolated human platelets stimulated by collagen. Stimulation of the platelets with thrombin also promoted tyrosine nitration, although to a lesser extent. Both processes could be significantly inhibited by the presence of high concentrations of the NOS inhibitor L-NAME. Several proteins were nitrated on tyrosine on stimulation of the platelets with collagen, and one was identified as being the vasodilator-sensitive phosphoprotein (VASP). VASP is a 46-kDa protein that permits the assembly of actin and facilitates the formation of focal adhesions (274). Actin and other protein constituents of the cytoskeleton are abundant in Tyr residues, which are essential for actin polymerization and protein-binding interactions. Ionic interactions and hydrogen bonding with cationic residues result from tyrosine nitration on actin. Furthermore, tyrosine nitration induces failure to control the growth of actin filaments, resulting in altered cell motility, attachment, intracellular transport, apoptosis, and loss of organ functions (14).

Cells respond to mechanical forces such as shear stress, the force generated by blood flow over endothelial cells by producing O_2^- and ·NO and by promoting gene transcription (84, 86). Potential sources of O_2^- and ·NO in the endothelium exposed to shear stress include the NOX isoforms, xanthine oxidase, eNOS, and the mitochondria. The induction of transcription of specific genes is mediated by the activation of the MAP kinases signaling cascades. Activation of JNK by shear stress involves the participation of protein kinases and G proteins (181, 132, 231). It was demonstrated that endogenous generation of $ONOO^-$ in endothelial cells exposed to shear stress is associated with the activation of JNK. Shear-stress–mediated JNK activation in endothelial cells was prevented by the use of the NOS inhibitor N^G-monomethyl-L-arginine, by the SOD analog Mn-TBAP, and by overexpression of Cu/Zn SOD (132).

As we discussed previously in this section, in addition to JNK, the ERK1/2 MAP kinases are also directly and indirectly activated by $ONOO^-$. In rat vascular smooth muscle cells stimulated with the hormone angiotensin II, ERK1/2 was nitrated and phosphorylated (activated). Nitration and phosphorylation of ERK1/2 was substantially inhibited by NOX inhibitors and by ROS and RNS scavengers. The use of 1400W, a selective inhibitor of iNOS, completely prevented the activation of ERK1/2. The authors concluded that angiotensin II, *via* activation of its receptor AT1, induces the production of RNS and ROS with potential formation of $ONOO^-$. The oxidant in turn targets and nitrates specific tyrosine residues on ERK1/2 and concomitantly induces tyrosine phosphorylation and activation of the kinases (299).

Basal endogenous tyrosine nitration on Src kinase and other Src PTKs was found in human pancreatic adenocarcinoma cells and in freshly isolated human platelets, respectively (253, 313). In addition, in adenocarcinoma cells, tyrosine nitration of Src was associated with upregulation of its kinase activity (253).

Endogenous tyrosine nitration was achieved after incubation of rat fibroblasts with diethylmaleate (DEM), an inhibitor of GSH synthesis. DEM promoted Akt activation independent of the activation of Src kinase and of the phosphorylation of the PDGFR. The authors suggested that tyrosine nitration in PDGFR occurs as a result of DEM treatment, whereas it is completely absent on cell stimulation with serum. The synergistic activation of Akt observed after simultaneous treatment of the cells with DEM and serum suggested the occurrence of two independent mechanisms: Serum stimulated tyrosine phosphorylation of some Tyr residues on PDGFR, whereas DEM stimulated tyrosine nitration of other residues. Both modifications can converge on and contribute to Akt activation (107).

Although compelling evidence regarding the signaling capacities of tyrosine nitration has accumulated, the experimental evidence on the reversibility of this posttranslational modification is still scarce.

A previous report described, in homogenate of rat tissues, a "nitrotyrosine denitrase" capable of denitrating exogenous nitrated albumin (187). The same group provided further evidence that the nitro group may be reduced nonenzymatically to aminotyrosine. The reaction was dependent on heme and thiols. It was shown that heme-containing proteins could mediate the reaction, which has its efficiency greatly enhanced by vicinal thiols (25). Reduction to aminotyrosine could facilitate its reduction and consequent removal by nitroreductases. Mammalian nitroreductases are well-described enzymes for nonpeptide

drugs (130); however, evidence for the existence of these enzymes for protein tyrosine denitration has not been established.

Mitochondria are capable of generating both ROS and RNS concomitantly; therefore, a number of mitochondrial proteins were found to be nitrated *in vivo* (15, 126, 370). Recent evidence indicated that mitochondrial proteins are nitrated and denitrated in the respiring mitochondria. Denitration was independent of protein degradation but depended on the O_2 tension. Mitochondria under periods of hypoxia–anoxia were capable of denitrating proteins. Reoxygenation reestablished protein tyrosine nitration in this model (204).

In conclusion, the reviewed experimental evidence provides strong support for the hypothesis of cooperation between both tyrosine nitration and tyrosine phosphorylation in cellular signaling events. Cooperation could take place by oxidative-based inhibition of PTPs or by nitration of specific tyrosine residues on tyrosine kinases, promoting conformational changes in the enzymes that will activate them. Furthermore, the degree of oxidative stress will determine the extent of such cooperation. We define two basic situations: In low metabolic stress conditions, a balance between tyrosine nitration/denitration and tyrosine phosphorylation/dephosphorylation dominates, and signaling events ensue. Conversely, excessive oxidative/nitrative stress leads to a protein nitration rate exceeding denitration, resulting in the formation of aggregates of extensively nitrated proteins that will be degraded by the proteasome system (Fig. 17).

IX. CONCLUDING REMARKS

Evidence has accumulated that ROS/RNS play a major role in the regulation of protein tyrosine phosphorylation. During the last decade of intensive research, it became clear that signaling pathways activated by RPTKs are interconnected with the production of redox active species. Activation of the nonphagocytic NOX isoforms and of the ·NO synthases is closely associated with ligand binding to growth-factor receptors and/or G protein–coupled receptors. Simultaneous generation of reactive species and increase in intracellular phosphotyrosine levels may represent the interplay between phosphorylation and thiol oxidation-dependent signaling at the level of PTP-mediated regulation of RPTKs. Direct effects on PTKs and on small G proteins also were observed. Therefore, it is accurate to state that signaling mechanisms relying on phosphorylation/dephosphorylation of tyrosine residues are controlled by the redox status of cells.

Growing experimental evidence covered in this review article has demonstrated the properties of NO and ROS as major regulators of such signaling networks. Protein modification by S-nitrosation of critical cysteine residues has been convincingly shown to regulate signaling magnitude and specificity (160). In addition, the concept of nitration of tyrosine residues as a noxious consequence of ·NO is leaving the dark side. Although several questions remain unanswered, several investigations suggest that tyrosine nitration may be involved direct or indirectly in the regulation of signaling proteins or at least cooperates with tyrosine phosphorylation (266). Protein structure–based studies coupled with a proteomics approach will be helpful in identifying signaling proteins modified by cysteine thiol oxidation and nitrosation, and tyrosine nitration. The relations between these oxidative-based protein modifications and protein tyrosine phosphorylation will strengthen our understanding of redox signaling.

In the past few years, signaling mechanisms have being under the scrutiny of the pharmaceutical corporations. The efforts at the industrial level are concentrated on two fronts: Identification of potential targets in the signaling pathways and devel-

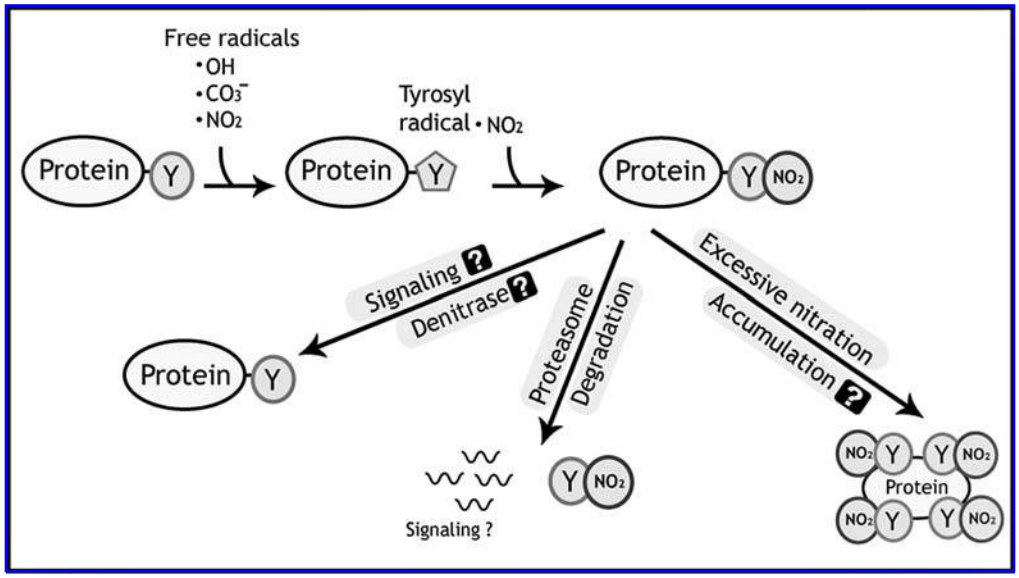

FIG. 17. Signaling by tyrosine nitration/denitration. Tyrosine nitration associated with low metabolic levels of stress may perform signaling functions. Putative denitrases keep the balance of nitrated/denitrated proteins. Higher levels of oxidative stress, resulting from pathological conditions, are associated with enhanced protein tyrosine nitration exceeding denitration. Under these conditions, nitrated proteins are accumulated and proteolytically degraded by the proteasome.

opment of inhibitors/activators of such targets. Indeed, many substances, including prodrugs such as GSH analogues, farnesyl transferase inhibitors, and specific neutralizing antibodies against growth factor receptors, had left the bench to be used in humans. Although some of them had not accomplished primary objectives in clinical trials, others have been used with relative success. Nevertheless, apart from failures and successes, the years to come will unravel more detailed information on the wiring of the pathway components and controllers to improve disease therapy and diagnosis.

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ABBREVIATIONS

BSO, Buthionine-L-sulfoximine; Csk, carboxy-terminal Src kinase; DEM, diethylmaleate; DSP, dual-specificity phosphatase; EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; FNIII, fibronectin type III; FAK, focal adhesion kinase; FAT, focal adhesion target; GSH, reduced glutathione; GSSG, oxidized glutathione; Grx, glutaredoxin; GPCR, G protein–coupled receptor; G-CSF, granulocyte colony-stimulating factor; GEF, guanine nucleotide exchange factor; HB-EGF, heparin-binding EGF; HSPG, heparan sulfate proteoglycan; IGF-1, insulin-like growth factor type 1; IR, insulin receptor; JNK, c-Jun N-terminal kinase; SIN-1, 3-morpholinosydnoimine; NAC, *N*-acetylcysteine; NOX, NADPH-oxidase; •NO, nitric oxide; NOS, nitric oxide synthase; SNO, nitrosothiol; GSNO, *S*-nitrosoglutathione; GSSG, oxidized glutathione; oxLDL, oxidized low-density lipoprotein; $ONOO^-$, peroxynitrate; PDGFR, platelet-derived growth factor receptor; PH, pleckstrin homology; Prx, peroxiredoxin; PMA, phorbol ester; PLCγ, phospholipase Cã; PI3K, phosphatidylinositol-3 kinase; Pyk2, prolinphospholipase Ce-rich tyrosine kinase 2; PTB domain, protein tyrosine binding domain; Prx, peroxiredoxin; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; RBD, Ras-binding domain; RNS, reactive nitrogen species; ROS, reactive oxygen species; RPTK, receptor-like PTK; RSK, ribosomal S6 kinase; SH3, Src-homologous type 3 domain; HS@ domain, Src-homologous type 2 domain; SMCs, smooth muscle cells; SOD, superoxide dismutase; TGF-β, transforming growth factor β; Trx, thioredoxin; UV, ultraviolet; VEGFR, vascular endothelial growth factor receptor; VASP, vasodilator-sensitive phosphoprotein.

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Address reprint requests to:
Hugo P. Monteiro, Ph.D.
Laboratory of Cell Signaling
Department of Biochemistry/Molecular Biology
Universidade Federal de São Paulo
Rua 3 de Maio 100; 4 andar
CEP: 04044-020
São Paulo, Brazil

E-mail: hpmonte@uol.com.br

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