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# Redox Regulation of Signal Transduction in Mammalian Cells

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**ABSTRACT.** This mini-review addresses the mechanism of ultraviolet-light-induced activation of receptor tyrosine kinases. The experimental approach into this mechanism revealed the existence of redox regulation of signal transduction in mammalian cells. It is postulated that, in addition to responsiveness to oxidative attacks from outside, redox regulation of specific redox-sensitive proteins likely represents an important physiological mechanism. BIOCHEM PHARMACOL **59**;1:35–41, 2000. © 1999 Elsevier Science Inc.

**KEY WORDS.** receptor tyrosine kinases; protein tyrosine phosphatases; UV response; substrate trapping; platelet-derived growth factor receptor; chemical cross-linking; in-gel phosphatase assay; ligand-independent activation of RTKs; tributyltin; photo-aging

## RECEPTOR TYROSINE KINASES AS PART OF FUNCTIONAL MULTIPROTEIN ASSEMBLIES

Signal transduction links the "outside world" with the cellular nucleus. The signal flow goes in both directions although the inward flow has been explored more than the outward flow. Part of this inward flow originates at cell surface transmembrane proteins, which exhibit the exquisite ability to deposit, upon ligand binding, a "start signal" on the inner side of the plasma membrane which sets off one or, most often, many cascades of intracellular communication. RTKs§ do this by "autophosphorylating" several tyrosines along their own intracellular tails. "Autophosphorylation" requires close proximity (dimerization/oligomerization) of receptor subunits so that mutual cross-phosphorylation can take place. The tyrosine phosphates form the cores of docking sites for several cytoplasmic proteins with either specific catalytic function, e.g. phosphoinositol 3-kinase and phospholipase C, or adaptor function, e.g. Grb2 and Sos [reviewed in Ref. 1]. The regulation of RTK activity involves not only the specific ligands, but the association with coregulatory molecules in the plasma membrane, e.g. signal-regulatory proteins [2], PTPs [reviewed in Ref. 3], and CD44 [4, 5; ¶]. While CD44 functions as a co-receptor required to make growth factors effective ligands, receptor regulation also occurs from the cytoplasmic side of the plasma membrane, e.g. by PTPs, Src, and protein kinase Cs, and yet unknown mediators of cross-talk with other receptors [see review in Ref. 6]. Just as it now emerges that intracellular signaling pathways are highly ordered in scaffolding matrices [e.g. 7], the receptors appear to be parts of large multiprotein assemblies organized in the plane of and below the plane of the plasma membrane.

## LIGAND-INDEPENDENT ACTIVATION OF RTKS

The data relevant to the issue of this mini-review are concerned with the activation of RTKs by toxic agents in the absence of ligand. Exogenous toxic attacks on biological organisms and cells, over wide dose ranges, elicit ordered and complex cellular responses [reviewed in Refs. 8, 9]. Cell cycle arrest, subsequent proliferation, and apoptosis figure among these complex reactions. The immediate expressions of such responses are changes in signal transduction. This is astonishing, as the agents considered here exhibit no discriminatory potency. Obviously, toxic agents will affect signal transduction and dependent reactions only if they alter the behavior of physiological macromolecules which are embedded in the intracellular communication network. "The toxic agents must talk in the language of the cell". A recently identified mechanism of toxicity neatly illustrates this point: dioxins can only cause toxic organismic effects such as thymus atrophy and immune toxicity if the cells of

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§ *Abbreviations:* RTK, receptor tyrosine kinase; EGFR, epidermal growth factor receptor; PDGFR, platelet-derived growth factor receptor; and PTP, protein tyrosine phosphatase.

¶ Rousseau V, Herrlich P and Ponta H, unpublished observations.

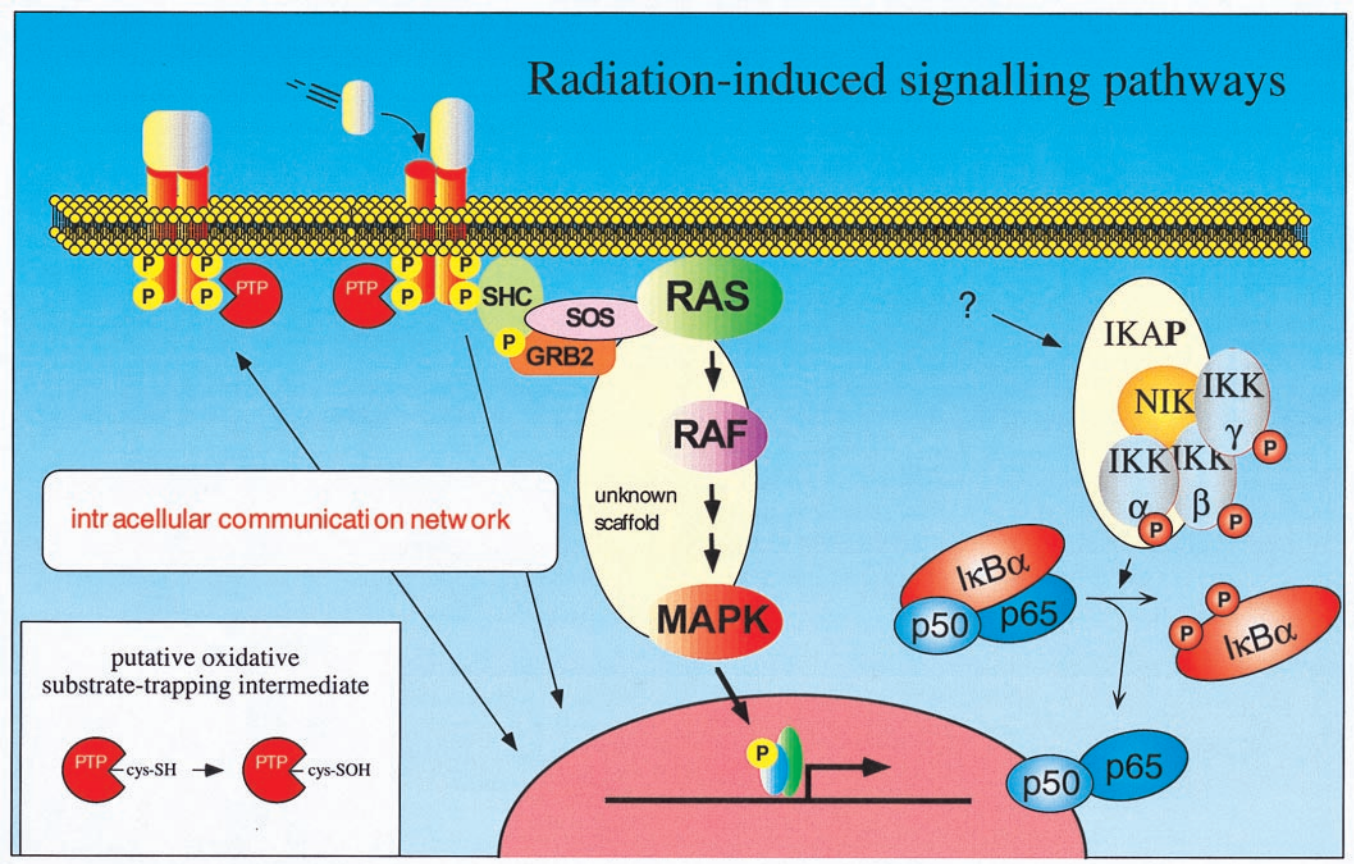


**FIG. 1. Radiation-induced signal transduction pathways. Only the nuclear factor-kappaB (NFκB) and Ras-Erk (extracellular-regulated kinase) pathways are shown. It is, however, clear that numerous other signaling pathways exist and that there is an enormous amount of cross-talk and signal flow in both directions, outside-in and inside-out. The primary target of UV radiation in the UV induced activation of NFκB is not yet known. Activation of RTKs by their ligands is symbolized here. UV, however, activates RTKs in a ligand-independent fashion and by inactivating PTPs. The insert shows the putative structure of oxidized and inactivated PTP. The sulphenic acid derivative of the active center cysteine may undergo subsequent reactions, e.g. to sulphinic acid. MAPK, mitogen-activated protein kinase; IKAP, IKK-complex-associated protein; IKK, IκB kinase; NIK, NF-kappaB-inducing kinase.**

the organism possess the aryl hydrocarbon receptor (AhR), a physiologically important transcription factor [10]. The AhR translates the presence of dioxins into a toxic reaction [11, 12]. As one of the several target genes activated by the AhR, AhR up-regulates transcription and synthesis of the cell cycle inhibitor p27Kip-1. In the absence of p27Kip-1 ($p27^{-/-}$ mice), only reduced thymic toxicity can be observed [13].

Ultraviolet or ionizing radiation, metal toxins, oxidants, and alkylating agents do not react with one specific "receptor" exclusively. Nevertheless, they do induce elaborate genetic responses [e.g. 14–16]. From the nature of the agents, it is to be expected that they react with many macromolecules, likely with a spectrum of target molecules that is peculiar to the agent. Most target molecules will, however, not be relevant for signal transduction. We will concentrate here on one class of target molecule which exquisitely serve to trigger signal transduction cascades: cell surface receptors (Fig. 1). Within about 15 sec and in the absence of their ligands, certain RTKs are activated by ultraviolet light of various wavelengths: (EGFR, [17]; PDGFR, [18]; insulin receptor locus [19]). Other ligand-dependent receptors are also activated in the absence of their physiological ligands: T-cell receptors [20], tumor necrosis factor receptors [21, 22], and CD95 [23, 24]. Due to the method of detection used, fairly high doses of UV were required for receptor activation, in some publications as much as 100 to 1000 J/m$^2$ UVC [e.g. 18]. Autophosphorylation of the EGFR by Western blot could, however, already be observed at 30 J/m$^2$, and EGFR-dependent signaling events occurred at even lower doses [17]. This is not too surprising as, in contrast to the bulk phosphorylation required for detection in Western blots, a small fraction of the receptors suffice for a full response of the signaling cascade. We will address here how receptor activation can be accomplished in the absence of ligand.

## RECEPTOR CLUSTERING

Does ligand-independent activation of RTKs resemble that elicited by the ligands? The best example of downstream signaling has been produced by looking at the insulin receptor [19]. This analysis left no doubt that UV-induced activation represented phosphorylation of those tyrosines

which are also modified in response to insulin. Introduction of negative-dominant receptor versions and experiments with specific inhibitors of the kinase function supported the hypothesis that the ligand-independent activation by UV (just like that induced by the specific ligands) occurred by cross-wise autophosphorylation of subunit dimers [17]. Interestingly, ligand-independent receptor activation with similar characteristics was also observed as the result of cross-talk of G-protein-coupled receptors with RTKs [25, 26]. In view of the complex extra- and intracellular interactions regulating RTK activity, a primary influence of UV on these interactions with secondary effects on the receptors was first considered. At least for the EGFR, ligand mimicry could, however, be excluded. A truncated spontaneously active EGFR which cannot bind EGF could be further stimulated by UV irradiation [18]. It is, by inference, more likely that the receptors themselves are modulated. The class of receptors activated by UV, is active in a dimer or oligomer configuration. Subsequent to activation, microscopic clustering can be detected, which is thought to precede internalization of the receptor. Interestingly, clustering of the tumor necrosis factor receptor and of CD95 could be induced by UV irradiation [21, 23]. The time points were taken at 5 and 60 min after irradiation, respectively. It is not yet clear whether the clustering is the primary event or the consequence of activation. If it were the primary event leading to receptor activity, the mechanism as to how UV triggers it would still be elusive.

With the idea in mind that UV may trigger dimerization of receptor subunits individually mobile in the plane of the plasma membrane, two attempts were made at testing this hypothesis.

1. Lowering the temperature to 10° or 4° prevented UVB-induced clustering of CD95 and CD95-dependent apoptosis [23, 27]. Treatment of isolated PDGFR containing membrane vesicles at 4° permitted spontaneous autophosphorylation to occur, which could be further increased only marginally by UV irradiation, while at 25° PDGFR activation depended on UV irradiation [28]. These two types of experiments seem contradictory. One interpretation is that low temperature stiffens the lipid membrane and prevents UV-induced CD95 oligomerization. In the case of the PDGFR, membrane stiffening would perhaps freeze the subunits in close proximity, such that they would be spontaneously active. As an alternative interpretation, low temperature may eliminate an enzymatic activity which regulates receptor activity. For instance, relevant phosphatases may not work at low temperature. If CD95-dependent signaling required the activity of a phosphatase, CD95 clustering and induced apoptosis would be prevented. In the case of the PDGFR, one could argue that the receptor is constantly active and regulation occurs through the rate of dephosphorylation.
2. A second equally puzzling result was obtained when attempting to detect UV-induced PDGFR cross-links. PDGFR vesicles or intact cells were treated with high doses of UV and the receptors (of the vesicles or of wheat germ agglutinin-enriched fractions from cells) were resolved by SDS–PAGE. No higher molecular weight bands indicative of receptor cross-links could be detected [28]. In order to stabilize a potentially induced non-covalent association, we added chemical cross-linkers that had previously been selected on the basis of their ability to cross-link receptor subunits after PDGF stimulation [29]. Indeed, covalent dimers could be recovered upon treatment *in vitro* or *in vivo* with PDGF, but no dimers were detected after UV irradiation [28], this despite the fact that autophosphorylation was of at least similar magnitude. All the autophosphorylation after UV irradiation *in vitro* was found on PDGFR monomers. Our tentative interpretation is that the PDGFR subunits are associated spontaneously, which suffices for effective activity. While the ligands induce a conformational change permitting cross-linking with the given chemical cross-linkers, UV does not induce this change. This conformational change may be important for PDGF- but not UV-dependent activation.

## INACTIVATION OF PTPS BY UV AND METAL TOXINS

The failure to detect UV-induced PDGF receptor subunit dimerization provoked a search for alternative targets. What happens to the lifetime of tyrosine phosphate on RTKs? The lifetime can be determined by measuring tyrosine phosphate after stopping further kinase activity by a specific inhibitor. The EGFR was totally dephosphorylated at all tyrosines within fractions of a minute [18]. The PDGFR *in vitro* [30] or *in vivo* [18, 31] was dephosphorylated with slower kinetics, i.e. $t_{1/2}$ about 15 min. Treatment with UVA, B, or C, with $H_2O_2$, or with organometal compounds such as tributyltin at subtoxic doses prolonged the lifetime considerably [18; and *]. The prolongation was sensitive to addition of excess *N*-acetylcysteine. Thus, UV irradiation and other toxins could either affect the structure of the receptors such that they could no longer be phosphorylated with the same fast kinetics, or inactivate the dephosphorylating enzymes. Both possibilities would result in a shift of the balance from a low level of autophosphorylation to a predominance of spontaneous kinase activity. This process was thiol-sensitive and could thus be prevented by excess *N*-acetylcysteine. That the dephosphorylating enzymes and not the receptors were the targets of inactivation was shown by separating a substrate fraction of membrane vesicles without phosphatase activity from one that contained no phosphorylated substrate but did contain phosphatase [18]. This latter fraction was vulnerable to UV treatment. When mixed together, the non-treated fraction successfully removed phosphate from tyrosines, whereas

* Klöhn PC, Knebel A, Zaucke F, Göttlicher M, Wilhelm D, Böhmer F, Rahmsdorf HJ, Krug H and Herrlich P, unpublished observations.

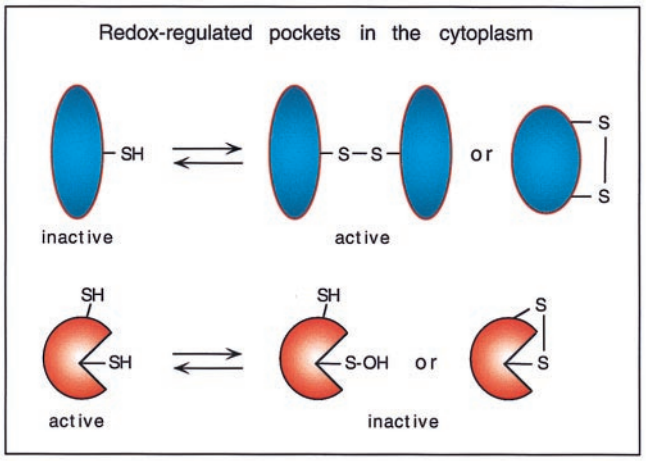


**FIG. 2. Redox regulation. Oxidation may activate or inactivate protein functions.**

UV-treated fractions could not. In these experiments, all phosphates of EGFR or PDGFR were always removed or their removal retarded. It is possible that several phosphatases acted in concert and were sensitive to inactivation by UV, or that one phosphatase acted as triggering enzyme or removed several phosphates consecutively. Phosphatase activity in the PDGFR vesicle preparations toward an artificial substrate was also clearly sensitive to UV irradiation [28]. *N*-acetylcysteine at mM concentration could prevent the inactivation and, in part, revert the inactivation when added after UV irradiation. Interestingly, several known PTPs, when overexpressed in cells, were also vulnerable to thiol-sensitive inactivation by UV or $H_2O_2$ [28], suggesting a common mechanism [32].

## A "SUBSTRATE-TRAPPING" CONFORMATION PRODUCED BY PTP OXIDATION

Thiol sensitivity and the action of $H_2O_2$ suggested an oxidative inactivation mechanism. Since all PTPs share a common amino acid motif in the active center of the enzyme with a catalytically essential cysteine in the middle of a groove fitting the tyrosine phosphate substrate [33], we speculated that this cysteine may be the target of oxidation. Based on the observation that a cysteine-to-serine mutation inactivated PTP catalytic activity but that the mutant PTP still bound fairly firmly to the substrate [34–37], we examined whether oxidation also caused a "substrate-trapping" conformation of the enzyme. Overexpressed tagged receptor-like PTPalpha could indeed co-precipitate the PDGFR if the cells had been treated with UV or $H_2O_2$ [28; and *], additional PDGF had little effect. The co-precipitation suggested that oxidized PTP structurally resembled the serine mutant. Presumably, the active center cysteine is oxidized to a sulfenic acid derivative (as was suggested in Ref. 32; see Figs. 1 and 2). The formation of a cys–cys–disulfide bond is less likely, although in Cdc25A, a tyrosine phosphatase involved in cell cycle regulation, the active center cysteine can be linked to a cysteine in close enough proximity, a result derived from electron density maps of some but not all crystals [38]. Metal toxins such as vanadate and tributyltin possibly complex and block the relevant PTP cysteine directly (Fig. 3). *N*-Acetylcysteine also counteracted the metal-dependent inactivation [unpublished data]. Although this mini-review has concentrated on PTPs interacting with RTKs, PTPs occur in several cellular compartments and are likely to be vulnerable to the same toxic attacks. Indeed, arsenite has been found to block dephosphorylation of c-Jun N-terminal kinase [39]. That UV or $H_2O_2$ alike produce a substrate-trapping conformation of PTPs requires that UV and—from the previous data on EGF receptor dephosphorylation discussed above—UV of various wavelengths generate oxidative intermediates which then react with the PTP cysteine. Unfortunately, no action spectra have yet been measured and the structure of the chromophore(s) absorbing the UV cannot be derived. Inhibitor studies and the introduction of cDNAs encoding enzymes of the oxygen metabolism yielded plausible arguments for the generation of singlet $^1O_2$ by UVA and of radicals and lipid peroxides by UVB [40, 41].

The firm interaction of oxidized PTP with its substrate opens up an interesting experimental avenue to identifying natural PTP–substrate relationships in the absence of overexpression. Endogenous PTPs can be converted into "trapping" derivatives and co-isolated with their cognate substrates. In a series of preliminary experiments, the concept proved promising. Antibodies to the EGFR precipitated, from a large panel of endogenous PTPs detected by in-gel phosphatase assay, individual bands very selectively (Fig. 4). In view of the fact that no physiological substrate has been identified for most of the 75-plus PTPs so far identified, and that all data on substrate specificity have thus far been obtained with strongly overexpressed PTPs, the approach of "reverse trapping" promises a significant advance.

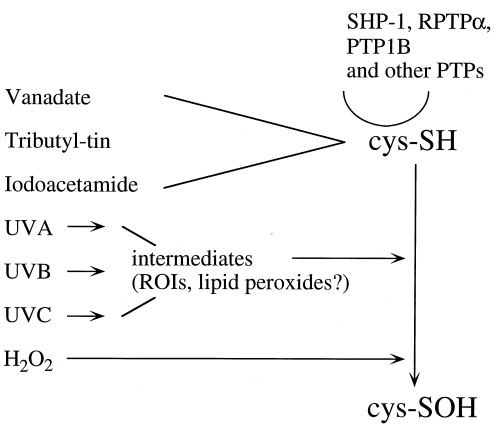


**FIG. 3. The catalytic center cysteine is attacked by many toxic agents. SHP-1, Src homology 2 domain tyrosine phosphatase 1; RPTPalpha, receptor-like PTPalpha.**

* Böhmer F and Herrlich P, unpublished data.

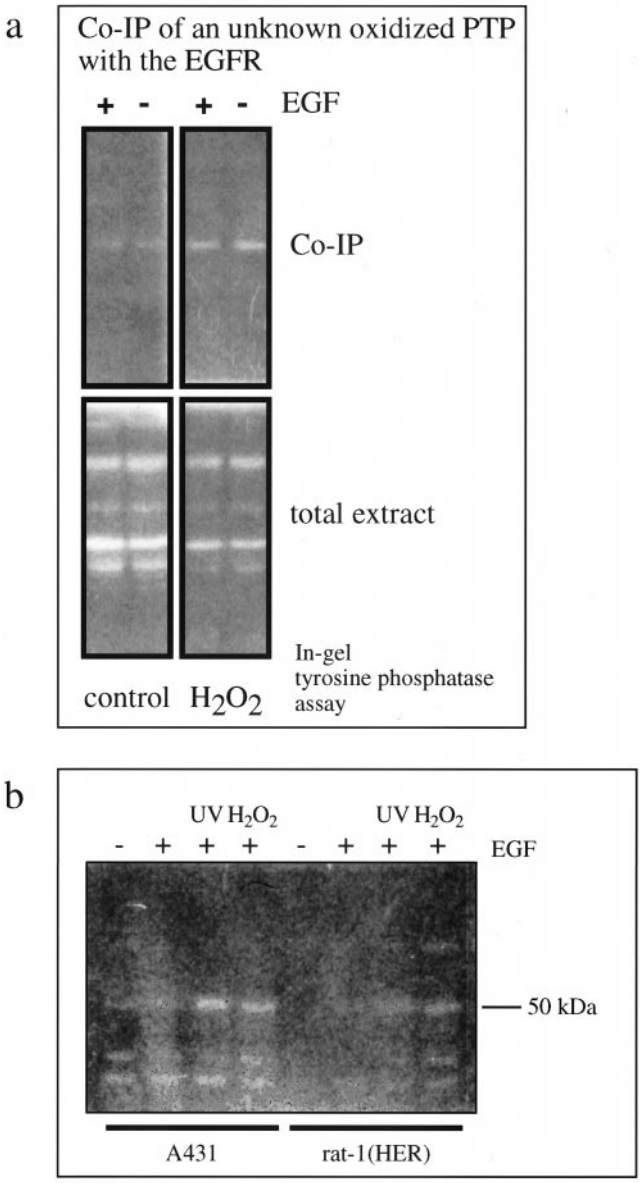


**FIG. 4. In-gel phosphatase assay for PTPs showing all testable (revertible after denaturation) tyrosine phosphatases of A431 cell extracts (a, lower panel) and a phosphatase of about 50 kDa co-precipitated with the EGF receptor (a, upper panel). Co-precipitation is strongly enhanced upon treatment of cells with $H_2O_2$. The co-precipitation has been reproduced in several types of cells (e.g. panel b) and can be triggered by UV irradiation of cells or treatment with $H_2O_2$. For methodology, contact the authors. HER, human epidermal growth factor receptor.**

Our preliminary data on "reverse trapping" generated another interesting result: after oxidation or UV treatment, the addition of EGF did not enhance the yield of co-precipitated PTP (Fig. 4). We interpret this to mean that upon inactivation of PTP, EGFR is spontaneously fully active such that additional EGF cannot further enhance kinase activity and thus binding of oxidized PTP. This is consistent with the hypothesis that the regulation of dephosphorylation and not of kinase activity is decisive for RTKs. How, then, would the ligand act if PTP were not inactivated? It is certainly speculative to assume that the ligand acted downstream of the binding of PTP to substrate and inhibited the subsequent dephosphorylation reaction.

## FROM INACTIVATION OF PTPS TO THE ORDERED UV RESPONSE

PTPs counteract tyrosine kinases. In addition to resetting the system after a stimulus, PTPs can be necessary for signal transduction [e.g. Ref. 42]. A toxic agent will inactivate PTPs according to the agent's accessibility to the PTP. Whatever the target PTPs, the cells will establish a new equilibrium between stimulatory and inhibiting pathways. The final outcome—a new set of expressed genes, proliferation, or apoptosis of cells—will reflect this new equilibrium.

## REDOX REGULATION, A GENERAL PHYSIOLOGICAL MECHANISM

To our knowledge, first proposals of a redox regulation in mammalian cells were based on the effects of external $H_2O_2$ and of thiol manipulations [43, 44]. The toxic agents addressed here possibly reveal physiological processes, and PTPs might be subject to redox regulation under physiological conditions. While the majority of cellular macromolecules are embedded in an excess of intracellular thiol groups, redox regulation may occur in microenvironmental pockets of reduced thiol availability. Physiological redox regulation would require the participation of redox enzymes. Indeed, for the activity of transcription factors, the participation of redox catalysts, e.g. thioredoxin and Ref-1, has been detected [45–47]. For the redox-sensitive PTPs, reactivation may also require a regulated and catalyzing partner protein. In the case of PTPs, we found loss of function by oxidation. Oxidation may, however, also activate proteins, for instance by immobilizing subunits through intermolecular disulfide bond formation such that they interact better or longer, or by a conformational change introduced through intramolecular disulfide bridges (Fig. 2). Previous work on bacterial redox regulation has offered several good examples of both activation and inactivation [48]. It is foreseeable that oxidative activation of proteins will also be detected in mammalian cells.

Taking the PTPs as candidates supports the existence of mammalian redox regulation. The attractiveness of PTPs as redox sensors lies in their extraordinary role in signal transduction. Oxidation of PTPs could be responsible for or contribute to tumor promotion and pathologies such as photo-aging of skin. This not only incorporates previous observations on oxidative steps in carcinogenesis, but provokes a new look at the mechanisms of redox processes in the development of disease.

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