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Forum Review

Glutaredoxin: Role in Reversible Protein *S*-Glutathionylation and Regulation of Redox Signal Transduction and Protein Translocation

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ABSTRACT

Reversible posttranslational modifications on specific amino acid residues can efficiently regulate protein functions. *O*-Phosphorylation is the prototype and analogue to the rapidly emerging mechanism of regulation known as *S*-glutathionylation. The latter is being recognized as a potentially widespread form of modulation of the activities of redox-sensitive thiol proteins, especially those involved in signal transduction pathways and translocation. The abundance of reduced glutathione in cells and the ready conversion of sulfenic acids and *S*-nitroso derivatives to *S*-glutathione mixed disulfides support the notion that reversible *S*-glutathionylation is likely to be the preponderant mode of redox signal transduction. The glutaredoxin enzyme has served as a focal point and important tool for evolution of this regulatory mechanism because of its characterization as a specific and efficient catalyst of protein-SSG de-glutathionylation (akin to phosphatases). Identification of specific mechanisms and enzyme(s) that catalyze formation of protein-SSG intermediates, however, is largely unknown and represents a prime objective for furthering understanding of this evolving mechanism of cellular regulation. Several proteomic approaches, including the use of cysteine-reactive fluorescent and radiolabel probes, have been developed to detect arrays of proteins whose cysteine residues are modified in response to oxidants, thus identifying them as potential interconvertible proteins to be regulated by redox signaling (glutathionylation). Specific criteria were used to evaluate current data on cellular regulation via *S*-glutathionylation. Among many proteins under consideration, actin, protein tyrosine phosphatase-1B, and Ras stand out as the best current examples for establishing this regulatory mechanism. *Antioxid. Redox Signal.* 7, 348–366.

I. OVERVIEW OF REVERSIBLE *S*-GLUTATHIONYLATION AS A MECHANISM OF REDOX SIGNAL TRANSDUCTION AND REGULATION OF PROTEIN TRANSLOCATION, AND THE CENTRAL ROLE OF GLUTAREDOXIN

POST-TRANSLATIONAL MODIFICATIONS of specific amino acid residues modulate the activity and/or localization of proteins that are involved in essentially every known cellular process. A number of enzyme-catalyzed, irreversible covalent modification reaction cascades, such as proteolytic cleavage of specific peptide bonds involved in the activation of zymogens, can constitute an efficient amplifier in response to biological challenges. These unidirectional cascades are utilized in blood clotting, complement fixation, and apoptosis. Other irreversible covalent modifications, such as *O*-glycosylation of the hydroxyl moiety of serine and threonine residues, *N*-myristoylation of glycine, *S*-farnesylation, and *S*-prenylation of cysteine residues, are involved in protein translocalization. However, for cellular regulation and signal transduction, reversible covalent modification is the mechanism of choice. This is due, in part, to its enormous capacity for integrating

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biological information and its capacity to fine-tune the activity of the target enzyme (15, 100). Thus, a specific criterion that distinguishes protein modifications as regulatory mechanisms is reversibility. Well established examples of these modifications are *O*-phosphorylation of the hydroxyl moieties on serine, threonine, and tyrosine residues, *S*-glutathionylation and *S*-palmitoylation of cysteine residues, and *N*-methylation, *N*-acetylation, and *N*-ubiquitinylation of lysine residues. The primary focus of this review is on reversible *S*-glutathionylation of cysteine as a regulatory mechanism, and the well characterized reversible *O*-phosphorylation mechanism is used both as an independent model for comparison and as a significant example of cross-talk among signal transduction mechanisms. The following is a brief overview of protein regulation via *O*-phosphorylation, followed by a focused discussion on cysteine modifications, especially *S*-glutathionylation. The latter more recently recognized and analogous regulatory mechanism involves specific mixed disulfide formation between cysteine residues and glutathione, *i.e.*, reversible protein-SSG formation.

Phosphorylation: prototype for reversible posttranslational modification as a regulatory mechanism in cellular signal transduction

Reversible phosphorylation is a key mediator of cellular signaling cascades by which a signal is amplified in an energy-efficient manner (15, 100, 110). It is the classic, most broadly understood mechanism of posttranslational modification, primarily involved in signal transduction and cellular regulation (52, 94, 108). The state of protein phosphorylation is controlled by protein kinases that catalyze the phosphorylation reactions and by protein phosphatases that catalyze the dephosphorylation reactions. Protein phosphorylation leads to alteration of the activity of the phosphorylated enzymes/proteins, and to translocation of proteins (52, 58). Phosphorylation often occurs at multiple regulatory sites along a single signal transduction pathway. For example, a given agonist could induce phosphorylation of IκB, the inhibitor of nuclear factor-κB (NFκB), at a specific site and target it for ubiquitin-mediated degradation, allowing the active NFκB to translocate from the cytoplasm to the nucleus and subsequently induce gene transcription (96). Additionally, direct phosphorylation of NFκB (p65 subunit) is necessary for DNA binding and gene transcription (96). Thus, phosphorylation can regulate gene transcription by NFκB both directly on NFκB and indirectly on upstream mediators. It should be pointed out that any direct activation of NFκB by phosphorylation is irrelevant unless it undergoes this translocation to the nucleus that enables it to carry out its function of promoting DNA transcription. Other factors whose phosphorylation coincides with a translocation event include SMADs (7), STATs (11), and actin (109).

Protein tyrosine phosphorylation constitutes a major mechanism of transmembrane signaling and also plays a key role in growth factor control. Agonist-mediated protein tyrosine kinase often propagates signals via tyrosine phosphorylation-induced protein–protein interactions mediated by phosphotyrosine-binding domains, such as the SH2 and PTB domains. This provides a mechanism to recruit proteins with phosphotyrosine-binding domains, such as phospholipase Cγ and phosphatidylinositol 3-kinase, to the activated membrane-localized receptors for signal processing. More recent findings show these phosphorylation-induced protein–protein interactions have been expanded from the phosphotyrosine-binding domain to phosphoserine- and phosphothreonine-binding domains. In addition, G proteins, such as Ras, are also involved in protein phosphorylation-mediated pathways. For example, the receptor protein tyrosine kinase-ras-mitogen-activated protein (MAP) kinase pathway leads to activation of MAP kinase, which could migrate into the nucleus to phosphorylate and activate transcription factors. The existence of multiple activation mechanisms provides cells with the ability to activate specific routes in response to a given signal.

Many proteins are regulated by more than one type of post-translational modification, which may or may not occur in the same subcellular location (31). For example, glycosylation and phosphorylation can occur simultaneously on different sites of a protein, such as with nuclear pore proteins and STATs (40, 49); or the modifications may occur in a mutually exclusive fashion at the same site of the protein, such as with c-Myc and RNA polymerase II (21). Analogous to these examples, a phosphorylated protein could also be *S*-glutathionylated. Conceivably, phosphorylation and *S*-glutathionylation could occur simultaneously because glutathionylation occurs on cysteine residues, whereas phosphorylation is selective for serine, threonine, and tyrosine residues. The addition of one modification could induce a conformational change to promote the reaction of the second modification. Alternatively, a mutually exclusive relationship could prevail if the two modifications resulted in steric hindrance, repulsive electrostatic interactions between the two negatively charged modifications, and/or conformational changes induced by one of the modifications that inhibit the second modification. One of the latter situations likely could be responsible for the observations, in separate studies, that actin in response to an extracellular stimulus in one case becomes deglutathionylated (111), and in another it becomes phosphorylated (109). The interplay between various modes of regulation is highly intriguing and represents the leading edge of the frontier of signal transduction studies. Future discoveries involving the exact localization of particular proteins at the time of specific modifications are needed to appreciate fully the significance of the interactions among the various modes of reversible covalent protein modifications linked to interactive regulatory pathways.

General considerations of modifications of protein-cysteine residues

Growth factors and cytokines appear to elicit intracellular oxidative signals that activate transduction pathways (36, 105) without necessarily changing the global redox status of the cell. Likewise, even exposure of hepatocytes to the oxidative burst of neutrophils did not alter the concentration of oxidized glutathione (GSSG) substantially in the hepatocytes [*e.g.*, Chai *et al*. (13)]. This lack of perturbation contrasts with the condition of overt oxidative stress where the cell is overwhelmed with excessive oxidants, diminishing substantially the intracellular ratio of reduced/oxidized glutathione

(GSH/GSSG) and leading to activation of various antioxidant defense systems. The normal intracellular milieu is a reducing environment with a GSH/GSSG ratio surpassing 100 (41, 63); however, this ratio approaches 1 under overt oxidative stress. Therefore, oxidative modifications of cysteine residues that occur under normal physiological redox signaling conditions need to be distinguished from those that occur under conditions that mimic pathophysiological oxidative stress, although the progression from one condition to the other likely represents a continuum.

There are many possible modifications of cysteine thiol groups upon reaction with reactive oxygen/nitrogen species (ROS/RNS). Analogous to the sulfoxidation of methionine (70), cysteine residues can be oxidized to the corresponding sulfenic (RSOH), sulfinic (RSO_2H) and sulfonic (RSO_3H) acids (20). Traditionally, sulfinates and sulfonates have been considered irreversible; however, recent findings revealed that sulfinates can be reduced via an enzymic process back to the thiol state (8, 14, 115). Sulfenic acid formation is known to be a reversible process; however, protein-sulfenates are typically unstable and become easily oxidized to sulfinates and sulfonates, or they can be captured by vicinal thiols or GSH to form intramolecular disulfides or protein-SSG mixed disulfides, respectively. Thus, protein-SSG formation, besides being recognized as a potential regulatory mechanism, is thought to be a homeostatic protective mechanism, preventing cysteine oxidation to irreversible forms (4, 12, 27, 64, 79, 106, 119).

S-Glutathionylation (protein-SSG formation) is likely the predominant mechanism of reversible cysteine modification, because it is promoted in an environment of relatively high concentrations of GSH (0.5–20 m*M*), *i.e.*, the physiological milieu (73, 76). Moreover, protein-*S*-glutathionylation is specifically reversed by the enzyme glutaredoxin (GRx), also known as thioltransferase (16, 47, 57, 79, 106, 117). Accumulation of protein-SSG has been reported in different cell types under a variety of oxidative conditions (13, 63, 90, 93). With many proteins, *S*-glutathionylation is inactivating, *e.g.*, the transcription factor nuclear factor 1 (NF1) (3), protein tyrosine phosphatase-1B (PTP1B) (4, 5), and phosphofructokinase (77, 118) are inactivated. With other proteins, glutathionylation can be activating, *e.g.*, HIV-1 protease (28) and microsomal glutathione *S*-transferase (24). Contrasting effects of *S*-glutathionylation for different proteins studied *in vitro* support the concept of reversible protein-SSG formation as a mechanism of regulation. For example, actin is deglutathionylated upon treatment of cells with epidermal growth factor (EGF), indicating that it is partially *S*-glutathionylated in the unstimulated cells (111). Furthermore, actin becomes both deglutathionylated (111) and phosphorylated (109) in response to an EGF stimulus. Actin membrane ruffling is only one example of how discrete localization and translocation may be linked to signal transduction. Thus, areas of concentrated signaling proteins have been identified in microregions of the cell. For example, lipid rafts of cells and postsynaptic density of neurons are localized regions of the membrane that house an abundance of signaling molecules (96 and 59, respectively). Hence, covalent modification and selective localization/translocation of a protein may represent alternative and/or synergistic mechanisms of regulation of its activity. Actin presents an excellent model that demonstrates regulation by phosphorylation, as well as *S*-glutathionylation and translocation, and it will be reviewed more thoroughly in section IV below, along with several other examples of *S*-glutathionylated proteins implicated in signal transduction pathways.

As increased attention has focused on cysteine modification as a means of signal transduction, several laboratories have developed methods for broadly identifying protein targets of *S*-thiolation (glutathione and cysteine) (6, 32, 38, 39, 65, 103, 106), *S*-nitrosation (55), and sulfenic acid formation (34). Section III reviews various approaches to discovering proteins with reversibly oxidized cysteine residues, especially *S*-glutathionylated proteins.

S-*Glutathionylation: a reversible posttranslational modification emerging as a cellular regulatory mechanism of signal transduction*

As described above, *O*-phosphorylation is the classic example of regulation of signal transduction pathways. By analogy, reversible oxidative modification of cysteine residues is considered a complementary means of regulation, and much recent evidence has implicated ROS as mediators of signal transduction for a multitude of growth factors and cytokines (105). Covalent modification of reactive cysteine residues on transcription factors and enzymes has emerged as a plausible mechanism for transducing the redox signal (22, 63, 79, 107). In particular, reversible *S*-glutathionylation is an attractive hypothesis based on the intracellular abundance and chemical nature of GSH and characterization of GRx (thioltransferase) as a specific and efficient catalyst of the deglutathionylation reaction (16, 47, 117). An abundance of data from biochemical studies supports *S*-glutathionylation/deglutathionylation as a homeostatic mechanism for protecting and restoring function to proteins altered by oxidative conditions. Direct evidence for reversible *S*-glutathionylation as a regulatory mechanism under normal physiological conditions is limited, but convincing (see section IV, below). In evaluating the evi-

Criteria for S-Glutathionylation as a Regulatory Mechanism

1. S-Glutathionylation must change the function of the modified protein.
2. S-Glutathionylation must occur within intact cells in response to a physiological stimulus.
3. S-Glutathionylation must occur at relatively high GSH/GSSG ratio, *i.e.* physiological conditions.
4. There must be a rapid and efficient mechanism for formation of specific proteins-SSG.
5. There must be a rapid and efficient mechanism for reversing the S-Glutathionylation reaction.

CHART 1. Criteria for regulatory mechanism. The chart lists criteria by which to evaluate data for reversible *S*-glutathionylation of specific proteins as a potential mechanism of regulation of their cellular functions. (See text for further explanation.)

dence for *S*-glutathionylation as a regulatory mechanism, we have identified the criteria in Chart 1 as necessary conditions.

Criterion 1 seems obvious, but the proteomic approaches described under section III (below) include numerous examples where cysteine-modified proteins are identified before corresponding changes in their activities and corresponding function have been documented. Also, many of the proteins have been identified after treatment of cells or isolated proteins with chemical oxidants; hence, criterion 2 focuses on responses to physiological redox stimuli, and includes the corollary that there must be a physiological endpoint that is modulated by the glutathionylation event(s). In contrast to evolution of the kinase-phosphatase story of regulation that was initiated through studies of the kinases, reversible *S*-glutathionylation as a regulatory mechanism has gained momentum via characterization of the enzymology of the deglutathionylation step (criterion 5; see elaboration below). Thus, catalysis of protein de-glutathionylation is clearly attributed to GRx, but very little is known about enzymatic mechanisms of formation of protein-SSG (criterion 4; see further discussion below). Simple reversal of the GRx catalyzed reaction, *i.e.*, transfer of the glutathionyl moiety from GSSG, is an unlikely intracellular mechanism because most protein-thiol redox potentials would require an unusually high GSSG concentration for formation of protein-SSG. Most cysteine residues have redox potentials such that 50% conversion of protein-SH to protein-SSG would require that the intracellular GSH/GSSG ratio would have to change from ~100 to ~1 (41). Criterion 3 is invoked to recognize that large global shifts in the intracellular redox potential are unlikely to occur under normal physiological redox signaling conditions, so other mechanisms for protein-SSG formation besides changes in the GSH/GSSG equilibrium need to be discovered (see below). A notable exception is c-Jun, which has an active-site cysteine residue with a redox potential of –0.27 V (K_{ox} = 13) (64). This means that c-Jun-SH is thermodynamically competent to be converted to 50% c-Jun-SSG by a GSH/ GSSG ratio of 13, *i.e.*, the change in GSH/GSSG from 100 to 13 corresponds to only about a 10% decrease in GSH concentration. However it remains to be seen whether the conversion to c-Jun-SSG is kinetically competent.

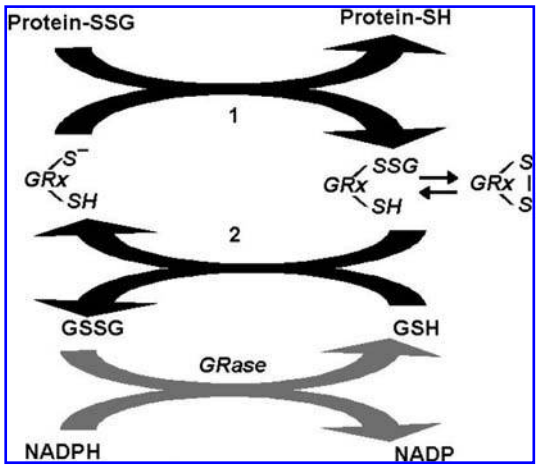
There are very few examples of *S*-glutathionylated proteins that are known to fulfill the criteria of Chart 1, so much additional work is required to establish reversible *S*-glutathionylation as a *bona fide* regulatory mechanism. For example, Klatt and Lamas (63) assembled a selected table of 23 proteins (reported during the period 1996–2000) that were shown to be *S*-glutathionylated under certain conditions. Of these 23, only one fulfills three or more of the criteria listed in Chart 1, namely, PTP1B. A more detailed discussion of PTP1B and other selected proteins (actin, protein kinases, etc.) is presented under section IV below, illustrating the application of the criteria of Chart 1. Also, proteins whose *S*-glutathionylation is consistent with a direct or downstream translocation event are discussed in detail in that section, including actin, protein kinase C (PKC), protein kinase A (PKA), and apoptosis signaling kinase-1 (ASK-1). The following briefly provides a more general overview.

In many reports, *S*-glutathionylation is characterized as an inhibitory modification whereby de-glutathionylation (chemical or enzymatic) reverses the modification and restores activity, *e.g.*, phosphofructokinase, (77, 118), carbonic anhydrase III (10), NF1, (3), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (71, 80), PTP1B (4, 5), protein kinase C-α (113), NFκB (87), creatine kinase (89), actin (111), protein phosphatase 2A (88), PKA (51), tyrosine hydroxylase (9), and mitochondrial complex I (104). However, in a number of cases, *S*-glutathionylation activates the protein of interest, *e.g.*, microsomal glutathione *S*-transferase (24), carbonic anhydrase III phosphatase-Cys186 activity (10), HIV-1 protease-Cys67 (27, 28), and matrix metalloproteinase (85). The list above is by no means comprehensive; however, it reflects the breadth of protein activities that can be modulated by reversible *S*-glutathionylation.

It is especially noteworthy that at least two of the proteins listed above can be glutathionylated at more than one cysteine residue, and their activities are modulated in a site-selective manner, namely, carbonic anhydrase III and HIV-1 protease. The latter example (HIV-1) protease illustrates an allosteric mechanism of inactivation involving interference with dimerization, in contrast to direct inactivation of an active-site cysteine residue (*e.g.,* GAPDH, PTP1B, etc.). The HIV-1 protease contains two conserved nonessential cysteine residues (Cys67 and Cys95) whose *S*-glutathionylation *in vitro* activates and inactivates HIV protease, respectively (27). *S*-glutathionylation of Cys95 (Cys95-SSG) appears to interfere with protease dimerization. Remarkably, GRx restores protease activity by deglutathionylating Cys95-SSG preferentially (28). Analogous to HIV-1 protease, human T-cell leukemia virus type 1 protease (HTLV-1) also has two conserved cysteines, Cys90 and Cys109 (29). *S*-Glutathionylation of HTLV-1 resulted in inactivation that was reversible with GRx (29). In fact, sequence comparison revealed at least one conserved cysteine (or methionine) within or near the regions of dimer interface in all retroviral protease genera, except those within the family of the gammaretrovirus family, suggesting a conserved regulatory role for reversible Cys-*S*-glutathionylation (or Met-sulfoxide) among the viral proteases (29). Thus, *S*-glutathionylation may regulate more long-lived interactions such as dimerization and membrane anchoring, in addition to regulating more transient modifications like disulfide formation at active-site cysteine residues involved in rapid signal transduction through the cell. The next section reviews the catalytic properties of the GRx enzyme that have put it at the focal point of considerations of reversible *S*-glutathionylation as a regulatory mechanism.

II. CHARACTERIZATION OF THE CATALYTIC PROPERTIES OF GRx: FOCUS ON REVERSIBLE GLUTATHIONYLATION

Until recently only one form of mammalian GRx (GRx1, or thioltransferase) was known, localized to the cytoplasm of cells and displaying 70–80% sequence identity across species and 25% homology to *E. coli* GRx1. Kinetic characterization (47, 78, 99, 117) established specificity of GRx1 for glutathionyl disulfides and delineated the mechanism (Scheme 1), coupled to GSSG reductase. Step 2 in Scheme 1



SCHEME 1. GRx catalytic mechanism. This scheme depicts the mechanism of GRx-catalyzed deglutathionylation of protein-SSG mixed disulfides, coupled to GSSG reductase (GRase), as derived from previous studies of the kinetics and substrate specificity of the enzyme. [See Mieyal *et al.* (79) 1995 for review.]

is rate-determining. Thus, substitution of various thiols for GSH and application of the Bronsted relationship (99) revealed two key features: (a) catalysis depends quantitatively on the unusually low pK_a (3.5) of the Cys22-thiolate serving as the leaving group, and (b) the nucleophilicity of GSH is enhanced in the enzymatic reaction (step 2).

Like other members of the thiol-disulfide oxidoreductase family, the GRx protein adopts a thioredoxin (TRx) fold, and the amino acid sequence of its active site is Cys-Pro-Tyr (Phe)-Cys. We characterized mutants of hGRx1, including (C7S, C25S, C78S, C82S)-GRx1 that retains only the C-22 active-site moiety (single cysteine, SC-GRx). Kinetics of the triple mutant (C7S, C78S, C82S)-GRx mimicked WT-GRx, but SC-GRx displayed a higher k_{cat} and lower K_M for GSH, revealing a key distinction between the thiol-disulfide oxidoreductase activities of GRx and TRx. The CXXC motif is required for TRx disulfide reduction activity, whereas GRx is a more efficient de-glutathionylation catalyst when the second Cys residue is replaced, preventing intramolecular disulfide formation (side reaction in Scheme 1) (117). SC-GRx was used to prepare NMR quantities of the stabilized GRx-SSG intermediate and solve its three-dimensional solution structure to identify key molecular interactions that constitute the basis for the glutathionyl specificity of GRx (117).

GRx has been shown to be 50,000–100,000 times more potent than GSH or dithiothreitol (DTT) alone for deglutathionylation of protein-SSG *in vitro* (28). Additionally, GSH alone did not reactivate NF1 (3), phosphofructokinase, pyruvate kinase, or glutathione-*S*-transferase, among others (79). In comparison with the TRx/TRx reductase system, the GRx/GSSG reductase system displays a 5,000-fold greater catalytic efficiency (k_{cat}/K_M) for deglutathionylating the prototype substrate Cys-SSG, which represents the common feature of all protein-SSG substrates without any steric constraints (16).

A second mammalian GRx (GRx2) with only 34% sequence homology to GRx1 was discovered (44, 74) by searching for expressed sequences analogous to the glutathionyl recognition site on GRx1. GRx2 is directed by leader sequences to the mitochondria and nucleus. The differential cellular localizations of GRx1 and GRx2 may be fundamentally important in modulating the steady-state *S*-glutathionylation status and corresponding activities not only of cytosolic and mitochondrial proteins that remain at those locations, but also of nuclear transcription factors that translocate from cytosol to nucleus. It appears that the mechanism of catalysis by GRx2 may differ from that by GRx1, including the remarkable ability of TRx reductase to reduce the GRx2-SSG intermediate efficiently in the absence of GSH (56).

Mechanisms of protein-SSG formation: potential novel role of GRx

As described above, evolution of the kinase-phosphatase story of regulation was initiated through studies of phosphorylation (kinases), followed by characterization of dephosphorylation (phosphatases). In contrast, reversible *S*-glutathionylation as a regulatory mechanism has gained momentum via characterization of de-glutathionylation (GRx). Although *S*-glutathionylation is a prevalent protein modification under oxidative stress, mechanisms of protein-SSG formation are not resolved.

Chart 2 lists potential mechanisms of protein-SSG formation that may occur spontaneously or be catalyzed by enzymes that are yet to be identified. Reversal of its typical deglutathionylation reaction, *i.e.,* GRx-catalyzed transfer of the glutathionyl moiety of GSSG to form protein-SSG (Scheme 1), is thermodynamically disfavored by the high GSH/GSSG ratio in cells (described above). Unless intracellular GSSG concentrations reach unusually high levels, GSSG is unlikely to be the substrate for protein-SSG formation based on typical redox potentials of cysteine residues (41), as described above (Chart 1, criterion 3). Consequently, *S*-nitrosoglutathione

Potential Modes of Protein-S-Glutathionylation

GSSG + Protein-SH	→	Protein-SSG + GSH
GSH + Protein-SOH	→	Protein-SSG + H_2O
GS$^{\cdot}$ + Protein-S$^{\cdot}$	→	Protein-SSG
GS-OH + Protein-SH	→	Protein-SSG + H_2O
GS-NO + Protein-S$^{\cdot}$	→	Protein-SSG + NO$^{\cdot}$
GS-NO + Protein-S$^-$	→	Protein-SSG + NO$^-$

CHART 2. Potential mechanisms of protein *S*-glutathionylation. This chart depicts various biochemical mechanisms by which protein thiol moieties could be converted to protein-SSG mixed disulfide adducts. (See text for further explanation.)

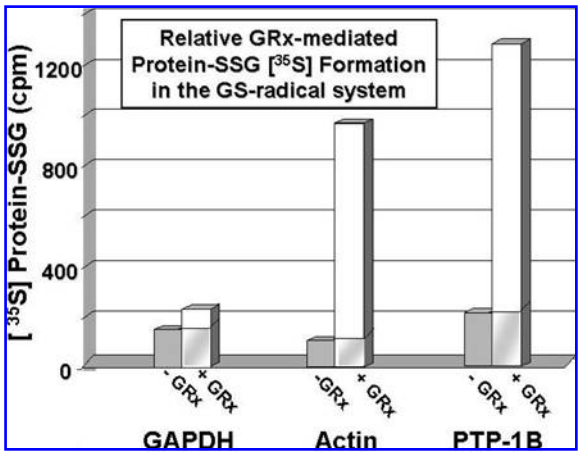


FIG. 1. Relative GRx-mediated *S*-glutathionylation of GAPDH, Actin, and PTP1B. GAPDH, actin, and PTP1B were compared as glutathionyl recipients in the GS-radical-generating system in the absence and presence of GRx. [For experimental details, see Starke *et al.* (101).]

(GS-NO) and GS• (GS-thiyl radical) have been proposed as alternative GS donors (22, 63, 80, 107). This prompted us to consider the known ability of thiyl radicals to be stabilized by formation of disulfide anion radicals (114), and we reasoned that GRx might react preferentially with GS• to form a GRx-S-S-glutathione disulfide anion radical (GRx-SSG•−). This concept was based on the unusually low pK_a (3.5) of the active-site cysteine of GRx (78, 99) and its selective stabilization of the glutathionyl moiety in the GRx-SSG catalytic intermediate (47, 79, 117). The GRx-SSG•− intermediate could facilitate transfer of the GS radical to form either GSSG or protein-SSG adducts. Indeed, GRx accelerates *S*-glutathionylation of several model proteins (GAPDH, PTP1B, and actin) in the presence of a GS-radical-generating system (101). We tested GSSG and GS-NO as glutathionyl donors for formation of GAPDH-SSG, but these were much less efficient substrates for GRx-mediated *S*-glutathionylation of GAPDH, requiring >10-fold higher concentrations than the GS-thiyl radical (101). Previously, GAPDH was shown to undergo GRx-reversible *S*-glutathionylation (71), and GS-NO was reported to be more efficient at glutathionylating GAPDH than GSH plus hydrogen peroxide (H_2O_2) (80).

Figure 1 shows that, in comparison with GAPDH, both actin and PTP1B are superior recipient proteins for GRx-mediated *S*-glutathionylation under GS-radical transfer conditions (101). Both actin and PTP1B are implicated in redox signaling, and both are subject to intracellular regulation via reversible glutathionylation (see section IV, below). Hence, these findings characterize GRx as a versatile catalyst capable of facilitating both *S*-glutathionylation and deglutathionylation of redox signal mediators via different mechanisms (101). Whether GRx or another enzyme is more important for catalyzing protein-SSG formation in cells remains to be elucidated. Indeed identification of enzymes responsible for catalyzing intracellular formation of specific protein-SSG intermediates represents one of the frontiers in characterizing redox signaling mechanisms.

III. PROTEOMIC APPROACHES TO IDENTIFYING PROTEINS WITH REVERSIBLY OXIDIZED CYSTEINE RESIDUES

Novel proteomic methods to detect proteins with reversibly oxidized cysteine residues have emerged recently, and the advantages and disadvantages of a number of these are reviewed briefly here, with an emphasis on protein-SSG detection. A variety of techniques have been reported (see below), utilizing such reagents as biotinylated GSH (Bio-GSH), biotinylated cysteine (Bio-Cys), biotinylated glutathione ethyl ester (Bio-GEE), biotinylated *N*-ethylmaleimide (Bio-NEM), NEM plus 5-iodoacetamidofluorescein (IAF), or L-[^{35}S]cysteine, to discover proteins with modified cysteines after treatment of cells under various oxidative conditions. In addition, Klatt *et al.* (65) demonstrated that immobilized GS-NO (*S*-nitrosoglutathione-Sepharose) could be used to identify proteins from cell lysates that are potential targets for GS-NO-induced protein-SSG formation.

Fluorescein-labeled thiol-selective alkylating agents

Two research groups have used thiol-directed fluorescein reagents to detect oxidized thiol proteins either using anti-fluorescein antibodies (116) or monitoring the fluorescence directly (6). Wu *et al.* (116) targeted cysteine residues that are especially reactive according to their low pK_a values by treating cell lysates with IAF at pH 5.5. They monitored protein oxidation (diminution of IAF reactivity) in lysates from A431 (epithelial) cells treated with H_2O_2. Although selectively identifying proteins such as PTP1B whose active-site cysteine thiol has a pK_a of ~5.5 (108), this technique did not detect abundant and typically identified oxidant-sensitive thiol proteins such as GAPDH [pK_a of reactive thiol = ~6.9 (42)] and actin [pK_a of reactive thiol = ~8.4 (111)]. Baty *et al.* (6) accomplished a more comprehensive and sensitive detection and characterization of reversibly oxidized proteins by first treating with NEM to block reduced thiols before treating with DTT, and then IAF. They used two-dimensional electrophoresis and documented changes in fluorescence intensity on paired two-dimensional maps derived from untreated Jurkat cells versus H_2O_2-treated or diamide-treated cells. The reproducibly detected protein bands were then subjected to proteolytic digestion and mass spectrometric analysis to identify them. The studies of Baty *et al.* (6) identified an extensive list of 57 H_2O_2-sensitive candidate proteins that might represent redox signaling intermediates; however, the technique is not able to distinguish among the possible DTT-reversible sulfhydryl modifications (*i.e.*, intramolecular disulfide, mixed disulfide, or sulfenate).

Biotin-adducted thiol-selective alkylating agents, and activation by GRx

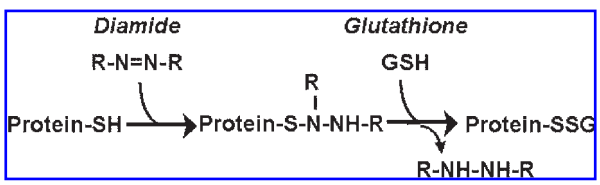
Two other studies used biotin attached to thiol-reactive agents to identify oxidant-sensitive thiol proteins. The biotin-thiol protein adducts were then isolated by binding to immobilized avidin. *N*-Biotinyl-*N*′-iodoacetyl ethylenediamine was

used at pH 6.5 in the absence and presence of 1 m*M* H_2O_2 to identify proteins such as protein disulfide isomerase and creatine kinase, whose reaction with the iodoacetyl derivative was prevented by oxidation of the respective cysteine residues (61). A potential difficulty with this study was the use of a single-bolus relatively high concentration of H_2O_2, which is likely not reflective of intracellular ROS signaling. In contrast, the study of Baty *et al*. (6) used concentrations of H_2O_2 as low as 20 μ*M*.

In a method developed by Lind *et al.* (72) [analogous to that of Baty *et al.* (6)], NEM was used to block any free sulfhydryls remaining within ECV304 cells after exposure to diamide (instead of H_2O_2). An important nuance of this study was that GRx (*E. coli* GRx3) (rather than DTT) was then added to the protein extract to generate free sulfhydryls. According to the documented specificity of GRx for deglutathionylating protein-SSG substrates (47), this procedure is interpreted to reactivate only those thiol proteins that were *S*-glutathionylated as a result of diamide treatment, and not intramolecular or intermolecular protein disulfides. The newly exposed free sulfhydryls were then bound to Bio-NEM. Avidin-Sepharose was used to isolate the Bio-NEM-bound proteins that were then separated by two-dimensional electrophoresis and identified by mass spectrometry. Forty-three proteins were identified by this procedure. This method offers the advantage of focusing on protein-SSG (deglutathionylation specific to GRx); however, the use of diamide is problematic. This cell-permeable reagent may mimic the formation of an activated thiol intermediate that would facilitate protein-SSG formation (see Scheme 2), analogous to what may occur in redox signaling (*e.g.*, protein-S-OH or protein-S-NO + GSH → protein-SSG). On the other hand this non-physiological oxidative stimulus may yield false positives because the reduced diamide moiety is a better leaving group.

Bio-GSH or Bio-Cys as direct reactants in thiol protein mixed disulfide formation

In studies reported by Eaton *et al.* (32, 33) rat hearts were perfused with *N*-biotinylated cysteine (Bio-Cys) (32) or *N*-biotinylated GSH (Bio-GSH) (33) and then subjected to the oxidative insults associated with ischemia and reperfusion, or diamide. Proteins captured by immobilized avidin were then released by reduction with DTT, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and sequence analysis of excised bands. Using Bio-GSH, one protein was identified by this procedure, namely, GAPDH (33). With Bio-Cys, 11 proteins were found to be *S*-thiolated. Although potentially useful for identifying thiol proteins that are especially sensitive to oxidative stress, there are several shortcomings of this approach for application to identifying redox signaling intermediates. First, Bio-Cys is an artificial substitute for the predominant nonprotein thiol in cells (GSH) and thereby may not accurately reflect enzyme-mediated processes that specifically utilize GSH. Secondly, as acknowledged by the authors, Bio-GSH is inefficient in crossing the plasma membrane and thus primarily labels membrane proteins (33). To circumvent this latter problem, other laboratories have used the ethyl ester derivative of Bio-GSH, *i.e.,* Bio-GEE, which is hydrolyzed intracellularly to Bio-GSH (51, 103).



SCHEME 2. Chemical activation of *S*-glutathionylation by diamide. This scheme depicts the activation of protein thiols by the oxidizing agent diamide, facilitating formation of protein-SSG mixed disulfides via reaction of GSH with the activated intermediate and displacement of the reduced form of diamide. (See text for further discussion.)

Hela cells were treated with the membrane-permeable Bio-GEE and oxidatively stressed with tumor necrosis factor-α (TNF-α) or H_2O_2, and proteins that were disulfide-linked (protein-SSG-Bio) were isolated with streptavidin agarose and released as protein-SH by treatment with DTT, separated via SDS-PAGE, and visualized with silver stain. Three proteins, GAPDH, annexin II, and TRx peroxidase II, were reported to be *S*-glutathionylated in response to TNF-α according to this procedure coupled to HPLC-mass spectrometry or HPLC–Edman sequencing. The identities of the proteins also were confirmed with specific antibodies, and they were shown to be radiolabeled when [^{35}S]cysteine was used to radiolabel intracellular GSH, instead of using Bio-GEE (103). Humphries *et al.* (51) used Bio-GEE in an analogous approach to study *S*-glutathionylation of PKA (see more complete discussion under section IV, below). The hydrolysis of Bio-GEE to give Bio-GSH within cells provides an efficient means of isolating and identifying proteins that may be *S*-glutathionylated in response to an oxidative stimulus. However, the attachment of the biotin moiety to GSH raises several concerns. Bio-GSH is not equivalent to GSH nor is it equilibrated with GSH intracellularly, so isolation of protein-SSG-Bio species cannot be used in a quantitative manner to reflect the steady-state level of *S*-glutathionylation of any given protein. In fact, as alluded to above, the biotin modification of GSH may interfere with enzymatic processes for formation and breakdown of specific protein-SSG intermediates. The alternative approach of radiolabeling the intracellular GSH pool has a different shortcoming (see next section).

Intracellular radiolabeled GSH from precursors: L-[^{35}S]cysteine/methionine

Pioneering work in the Thomas laboratory led to reports about a decade ago of a technique for documenting protein mixed disulfide formation in cells under oxidative stress conditions (13, 57, 106). According to this approach, cell-permeable radiolabeled precursors of GSH (^{35}S-labeled L-cysteine or -methionine) were used to radiolabel intracellular GSH. Cells were pretreated with cycloheximide to inhibit protein synthesis so that the intracellular radiolabel would occur predominantly as [^{35}S]GSH and not be incorporated into newly synthesized proteins. In some cases, the specific radioactivity of the intracellular GSH was enhanced by also pretreating cells with buthionine sulfoximine (inhibitor of glutathionine synthetase) to temporarily diminish the content of unlabeled

GSH. Radiolabeled protein disulfides were identified on non-reducing SDS-PAGE gels according to DTT-dependent loss of radiolabel. The DTT-releasable radioactive moieties were identified as ≥95% GSH by HPLC analysis. Certain radiolabeled proteins were identified by western blot analysis. This approach is established as a specific method for identifying protein-SSG formation intracellularly, and it has been used to document that GRx is the main catalyst of deglutathionylation of protein-SSG in mammalian cells (16, 57). Recently, the technique was advanced further by applying two-dimensional electrophoresis and coupling it to mass spectrometry for broader identification of specific proteins (38, 39). Thus, 32 proteins were identified to be *S*-glutathionylated in response to an oxidative stress (H_2O_2, diamide, or menadione) in isolated hepatocytes, HepG2 cells, or T-cell blasts, using L-[^{35}S]cysteine. Cell lysates were separated into protein clusters on two-dimensional gels, excised radiolabeled spots were trypsinized, and individual proteins were identified according to their mass spectra in comparison with known protein sequences. This approach has the distinct advantage of documenting protein-SSG formation directly with radiolabeled unmodified GSH in a manner that could be adapted to quantitative analysis of specific protein-SSG intermediates according to the specific radioactivity of the intracellular GSH. This technique obviates the questions raised (above) about potential interference (by the biotin-GSH derivatives) with enzymatic reactions involving transfer of the glutathionyl moiety. However, a new concern arises when this approach is considered for application to studies of physiological signaling events. Namely, inhibition of protein synthesis by cycloheximide may distort normal cell functions by depleting vital proteins that have rapid rates of turnover. Changes in *S*-glutathionylation status of certain proteins could reflect the artificial interference by cycloheximide rather than being indicative of a redox signaling cascade initiated by the effector molecule.

Synopsis of approaches to identifying specific protein-SSG intermediates

As described above, efficient methodology is rapidly emerging that detects proteins with oxidant–sensitive cysteine residues, the oxidative insult being produced in various ways, including diamide, menadione, H_2O_2, ischemia/reperfusion, and TNF-α. Many of the approaches do not distinguish protein-SSG formation from the other possible types of oxidized cysteine modifications, and there are many shortcomings that preclude definitive and quantitative assessment of the role of the identified proteins in signal transduction. Future studies are needed to evolve this methodology into an efficient and accurate detection for *S*-glutathionylated proteins within cell signaling pathways in both the absence and presence of an oxidative signal. In order to focus the proteomic approach on identifying possibly low abundant protein-SSG intermediates under physiologically relevant redox signaling conditions, it seems that an effective protocol could be evolved by combining the key features of a number of the techniques reviewed above with other experimental approaches. The following stepwise scheme was synthesized from the reports of Barrett *et al.* (4, 5), Baty *et al*. (6), Lind *et al.* (72), and Gitler *et al.* (43). In order to focus on signaling intermediates, a physiological stimulus (rather than chemical oxidant) should be used, *e.g.,* growth factor, cytokine, etc., and agents like cycloheximide that may interfere with normal cellular functions should be avoided. GRx (rather than DTT) should be used to free the modified cysteine residues after NEM block of reduced thiols. The freed thiols then can be reacted with IAF or *N*-iodoacetyl-[^{125}I]iodotyrosine (IAIT). The latter reagent can be made with very high specific radioactivity to detect low abundant proteins, and it has been characterized by Gitler *et al.* (43) as uniquely reactive with protein thiols, *i.e.*, it is reported to selectively label thiol proteins even in the presence of 20 m*M* DTT. Use of [^{125}I]IAIT would also facilitate quantitative analysis of the degree of *S*-glutathionylation of a signaling intermediate. Specific proteins should be isolated from cells by quantitative immunoprecipitation from control and treated cells, and their protein-SSG status confirmed by mass spectrometry (*i.e.* the molecular mass of the peptide bearing the *S*-glutathionylated cysteine should increase by 306 Da, corresponding to the glutathione moiety). Anti-glutathione antibodies are emerging as a tool for identifying protein-SSG, and they have been used effectively in studying the *S*-glutathionylation status of actin (see discussion under section IV, below). However, there is no substitute currently for either mass spectrometry or direct radiolabeling with [^{35}S]GSH to document actual glutathione adduction to the protein of interest. The stepwise protocol is as follows:

1. Use physiological stimulus.
2. Block free thiols with NEM.
3. Reduce with GRx to convert protein-SSG to protein-SH.
4. Treat with IAF or IAIT.
5. Identify proteins by mass spectrometric analysis.
6. Produce specific antibodies to the identified proteins.
7. Immunoprecipitate the protein(s) of interest from NEM-treated cell lysates after redox stimulation.
8. Confirm ΔMW = 306 to validate protein-SSG.

IV. EXAMPLES OF REGULATION OF REDOX SIGNALING BY GRx, INVOLVING REVERSIBLE *S*-GLUTATHIONYLATION OR INTERPROTEIN COMPLEX FORMATION

This section is focused on analysis of a few specific examples that illustrate the current status of knowledge of redox regulation involving reversible *S*-glutathionylation and GRx. The examples are discussed according to the relative weight of evidence supporting intracellular regulation.

PTP1B

The protein tyrosine phosphatase (PTP) family consists of enzymes either specific for tyrosine alone or acting on tyrosine, serine, and threonine (108). PTP activity is regulated in part by subcellular localization whereby the C-terminus directs PTPs to the outside of the endoplasmic reticulum and by

posttranslational modifications of C-terminal serine and threonine phosphorylation (108). Oxidation of both cytosolic and receptor-like PTPs (RPTP) has been demonstrated. RPTPα has two PTP domains that are distinguishable in oxidation susceptibility (86). Oxidation of PTP1B, the human tyrosine-specific phosphatase, has been one of the more extensively studied phosphatases, and it serves as a model for the family as a whole.

Growth factors and cytokines elicit transient tyrosine phosphorylation of intracellular proteins in conjunction with generation of ROS. Pursuing the basis for the change in tyrosine-phosphate status, Lee *et al.* (68) learned that EGF stimulation of cells leads to ROS-mediated reversible deactivation of PTP1B *in situ*. To simulate the oxidative modification cycle, they treated PTP1B with H_2O_2 on ice, then examined relative reactivation by the TRx and GRx systems. The somewhat more rapid reactivation by TRx was interpreted to mean that the active-site cysteine of PTP1B was converted to a sulfenic acid by H_2O_2. However, it is difficult to extrapolate these studies to the intracellular situation, because the oxidative deactivation of PTP1B was carried out in the absence of GSH, which is abundant in all cells during exposure to oxidizing agents. In a subsequent study, treatment of isolated PTP1B with H_2O_2 at room temperature gave largely irreversible inactivation, whereas superoxide generation produced reversible oxidation of the active-site Cys215 to a sulfenic acid intermediate, which was shown to react with GSH to give the more stable mixed disulfide PTP1B-SSG (4). Furthermore, part of the PTP1B isolated by immunoprecipitation from cells stimulated with EGF was documented by mass spectrometry to be glutathionylated at its active-site Cys215 (4). Treatment of isolated PTP1B with a relatively high concentration of GSSG (or GSH + diamide) gave a form of PTP1B-SSG that could be reactivated by GRx, albeit slowly [second-order rate constant $k < 0.03$ min^{-1} (5)]. Collectively, these data favor reversible *S*-glutathionylation as a probable mechanism of intracellular regulation of PTP1B activity, fulfilling criteria 1–3 of Chart 1 (above). Thus, PTP1B-SSG is formed intracellularly, changing the function of the enzyme by preventing formation of the catalytic cysteine-phosphate intermediate. This functional change occurs in response to a physiological stimulus (EGF) without substantial disruption of the global GSH/GSSG ratio.

However, the fine control expected in signal transduction would seem to require both selective and efficient formation and breakdown of PTP1B-SSG within the intracellular milieu. Accordingly, we set out to more closely simulate intracellular signaling events by studying reversible deactivation of PTP1B in the presence of GSH and the simultaneous presence of a selectable ROS-generating system (xanthine + xanthine oxidase ± catalase ± superoxide dismutase) and either the GRx or TRx enzyme systems.

This approach distinguished GRx as the likely controlling factor of PTP1B activity under ROS-generating conditions in the presence of GSH. Reversibility of PTP1B deactivation was dependent on the specific ROS generated. GRx effectively protected PTP1B when superoxide was the oxidant (+ catalase), but not with H_2O_2 (+ superoxide dismutase) (Fig. 2) (102). The TRx system was ineffective in both cases. From the concentration dependence of the protective effect of GRx, we estimated the second-order rate constant k to be >0.5 min^{1}, ~20 times higher than that seen with reactivation of the GSSG-treated enzyme (5). This study simulates intracellular oxidative signals in the presence of GSH and enzyme systems that reverse different types of cysteine-SH modification. For PTP1B, it appears that reversible *S*-glutathionylation involving GRx is the favored reaction. Nevertheless, additional work is necessary to document the enzymes that catalyze formation and breakdown of PTP1B-SSG *in vivo.*

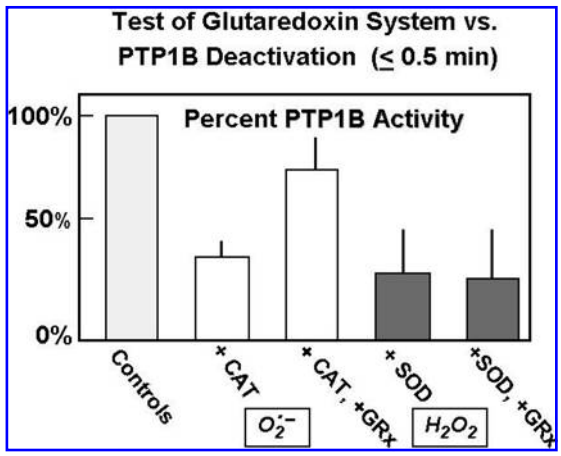


FIG. 2. Modulation of PTP1B activity by differential ROS generation: effect of the GRx system. [See Starke *et al.* (102)]. PTP1B deactivation/protection was carried out in reaction mixtures (50 µl total volume) containing: reaction buffer (50 m*M* imidazole, 150 m*M* NaCl, 1 m*M* EDTA), 3 ± 0.3 µ*M* PTP1B, 1 ± 0.1 µ*M* xanthine oxidase (XO), 0.5 m*M* GSH, 0.2 m*M* NADPH, 4 units/ml GSSG reductase, in the absence or presence of GRx (2 µ*M*) and either catalase (CAT) (0.5 mg/ml) to scavenge H_2O_2 and favor superoxide ($O_2^{\bullet-}$) formation, or superoxide dismutase (SOD) (5 mg/ml) to scavenge superoxide and favor H_2O_2 formation, or both (control). Reactions were initiated by adding xanthine (X) to 2 m*M*. Several aliquots were taken during the first minute after xanthine addition and diluted into assay mixture. Then 2 µl of 100 m*M* DTT was added to the remaining reaction solution, and incubation was continued for 5 min. Values for PTP1B activity for the DTT-treated X/XO/CAT ± GRx samples overlapped with those for the two types of control, namely, "minus xanthine" and "CAT + SOD," indicating recovery of full activity. Accordingly, all of these values were pooled to calculate the mean control values (180 ± 5 mA_{405nm}/min [n = 41]), defined as 100%.

Actin

Growth factors are known to initiate signal transduction pathways that induce actin rearrangements, such as ruffling at the periphery of the cell membrane. Membrane ruffles house concentrated tyrosine-phosphorylated proteins and active receptors, and therefore they are key to signal transduction pathways (109). Actin has been shown to polymerize into filaments, translocate from a uniform cytosolic distribution to the cellular periphery, and rearrange into membrane ruffles in response to EGF in A431 cells (111) and in response to fibroblast growth factor (FGF) in NIH3T3 cells (112). As seen in Fig. 3, confocal microscopic images reveal yellow fluorescent protein-fused actin concentrated at the periphery after EGF stimulation (panel B) (111).

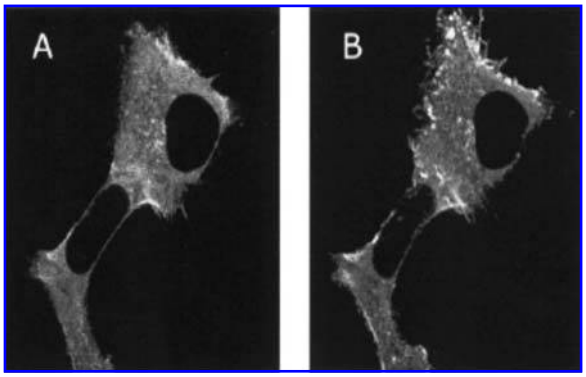


FIG 3. Translocation of actin in response to EGF in A431 cells. Confocal microscopic images reveal yellow fluorescent protein-fused actin concentrated at the periphery after EGF stimulation (panel B). [For detailed description, see Wang *et al.* (111).]

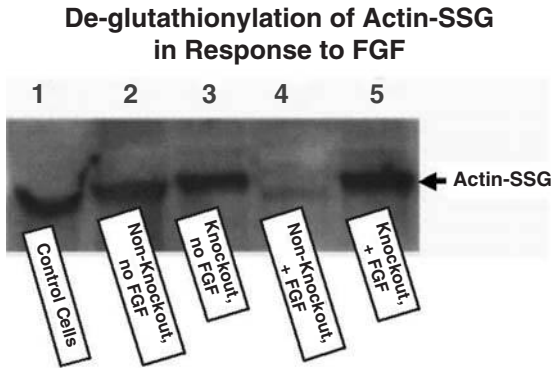


FIG. 4. Regulation of actin glutathionylation by GRx. Specific knockout of GRx (GRx1) in NIH3T3 cells by tetracycline-inducible RNAi abolished FGF-mediated deglutathionylation of actin (see lane 5 vs. lane 4), confirming that GRx1 is the intracellular catalyst. [See Wang *et al.* (112) for further details.]

Actin is perhaps the best example of physiologically relevant regulation by *S*-glutathionylation. In contrast to PTP1B, EGF treatment of A431 cells leads to deglutathionylation of actin despite an increase in intracellular ROS (111). Therefore, we investigated the potential biological consequences. Western blots with anti-GSH antibodies and mass spectrometry showed that, under normal cellular conditions, a portion of G-actin is *S*-glutathionylated at Cys374. This likely inhibits polymerization into F-actin, as deglutathionylation leads to an increased rate of polymerization. The deglutathionylation is catalyzed by GRx. Thus, addition of GRx to immobilized actin-SSG showed efficient transfer of the GS-moiety to GRx according to mass spectrometry. Cadmium, an inhibitor of intracellular GRx (16), inhibited de-glutathionylaton of actin in cells. Furthermore, specific knockout of GRx1 in NIH3T3 cells by tetracycline-inducible RNAi abolished growth factor-mediated actin polymerization, translocalization to the cell periphery, and membrane ruffling (112). These studies support the conclusion that reversible *S*-glutathionylation of actin by GRx contributes to the regulation of the cellular functions of actin. According to the data described above, redox modification of actin fulfills at least four of the criteria in Chart 1 (criteria 1, 2, 3, and 5) for reversible *S*-glutathionylation as a regulatory mechanism. Although the kinetics of deglutathionylation of actin-SSG by GRx have not been studied directly, the facile transfer of the glutathionyl moiety from actin-SSG to form GRx-SSG suggests efficient catalysis of deglutathionylation. And the lack of intracellular deglutathionylation of actin in response to FGF when GRx1 was knocked out confirms that GRx is the intracellular catalyst (criterion 5; see Fig. 4, lane 5 versus lane 4).

The enzymatic basis for formation of actin-SSG (criterion 4), however, remains a mystery. Under GS radical-generating conditions, GRx was shown to mediate actin-SSG formation (see Fig. 1 under section II, above), but GRx appears not to be required for maintenance of the steady-state level of actin-SSG, at least in NIH3T3 cells (see lanes 2 and 3 in Fig. 4). There are a number of alternative interpretations for the lack of change in the actin-SSG steady state in the absence of intracellular GRx1. If formation of actin-SSG is catalyzed by another cytosolic enzyme, then the steady-state actin-SSG content would be increased in the absence of GRx-catalyzed deglutathionylation. This suggests that actin-SSG may be preformed in the endoplasmic reticulum and then conformationally shielded until the FGF signal is transmitted. Thus, regulation of actin function by *S*-glutathionylation of Cys374 decreases the rate of actin polymerization most likely due to a change in protein conformation (25, 111). Clearly, additional studies are necessary to understand this phenomenon completely and to relate it to other mechanisms of actin regulation.

EGF is known to activate intracellular kinases that phosphorylate serine residues of actin (109), and actin polymerization is regulated by protein kinase phosphorylation (84). Phosphorylation of actin by PKC and PKA increase and decrease actin polymerization, respectively (84). PKC-β1, specifically, has been shown to stabilize F-actin in addition to preventing NFκB activation (1). Thus, PKA and PKC, which are both *S*-glutathionylated also (see below), add yet another layer to regulation of actin involving phosphorylation and *S*-glutathionylation.

Moreover, many proteins interact with actin to enhance its organization, such as actin-cross-linking proteins that connect actin filaments to each other and membrane-microfilament binding proteins that connect the actin cytoskeleton to the cell membrane. One group of multifunctional heterotetrameric proteins called annexins serve as physical mediators between actin and the plasma membrane (35). In particular, annexin II is distinct in its ability to exist as a monomer or tetramer, and binding of tetrameric annexin II to actin *in vitro* is attributed to the bundling properties of F-actin (35). Negative regulation of annexin II binding activity includes phosphorylation of Tyr23 or Ser25 (50). Additionally, annexin II has four cysteines, Cys8, Cys132, Cys261, and Cys334, that could be targets for sulfhydryl modifications such as *S*-glutathionylation. Indeed, glutathionylation of Cys8 and Cys132 of tetrameric annexin II causes an inhibition of annexin binding activity that is reversible by GRx (12). At the cellular level, *S*-glutathionylation of annexin II has been identified in

HeLa cells after stimulation with TNF-α (103). Collectively, these data provide a basis for proposing that *S*-glutathionylation regulates actin both directly (as described above) and indirectly through annexin II. Other cytoskeletal proteins besides actin (vimentin, myosin, tropomyocin, cofilin, and profilin) also have been shown to be glutathionylated in human T-cell blasts in response to oxidants (38). Thus, understanding the regulation via *S*-glutathionylation of actin and other cytoskeletal components is likely to evolve as another example of a classic mode of regulation of translocation and signal transduction widespread throughout the cell.

NF1

Transcription factors are downstream effector proteins of signaling cascades. For example, c-Jun, cyclic AMP (cAMP) response element binding protein (CREB), and NFκB are regulated by a series of phosphorylation events leading to modulation of gene expression events resulting from the initial signal. Transcription factors like CREB can be found in the nucleus before activation, or like NFκB they can be activated in the cytoplasm and then translocated to the nucleus. The family of transcription factors known as NF1 is important for cellular transcription and adenoviral DNA replication (2). NF1 can be modified by various posttranslational events, namely, *N*-glycosylation, phosphorylation, and glutathionylation, but only the latter alteration has been shown to influence the DNA-binding affinity (2, 3). Consideration of *S*-glutathionylation of NF1 serves as a model for other transcription factors, such as NFκB and c-Jun, that have been shown to be glutathionylated *in vitro* (64, 87).

It was discovered that the DNA-binding activity of the NF1 family of transcription factors was inactivated by oxidation *in vitro* (2). This sensitivity to oxidation required the presence of a single oxidation-sensitive cysteine residue that is conserved within the DNA-binding domains of all NF1 proteins from *C. elegans* to humans. Mutation of this cysteine residue creates NF1 proteins with apparently normal intrinsic DNA-binding activity, but the DNA binding of the mutant proteins is no longer sensitive to oxidative inactivation *in vitro*. This finding suggests a regulatory function for the oxidation-sensitive cysteine residue analogous to the situation for HIV-1 protease (described above), which has two highly conserved cysteine residues that are not required for basal activity, but their modification by *S*-glutathionylation alters the activity of the protease in a reversible fashion (28).

Oxidation of the single cysteine residue of NF1 in the presence of GSH likely forms NF1-SSG *in vitro* or *in vivo,* and this treatment inactivates its DNA-binding activity as shown by electrophoretic mobility shift assay. Therefore, we assessed the ability of GRx to restore DNA-binding activity to NF1 that had been oxidized by diamide in the presence of GSH (3). We found that GRx catalyzes restoration of binding in a concentration-dependent fashion as illustrated in Fig. 5. Treatment of HeLa cells with buthionine sulfoximine, a known inhibitor of GSH synthesis, potentiated the oxidative inactivation of NF1, probably resulting from impaired turnover of GRx due to limiting GSH. Inclusion of *N*-acetylcysteine (a precursor of intracellular GSH) in the culture medium following diamide treatment and removal increased the extent of recovery of NF1 DNA-binding activity. These

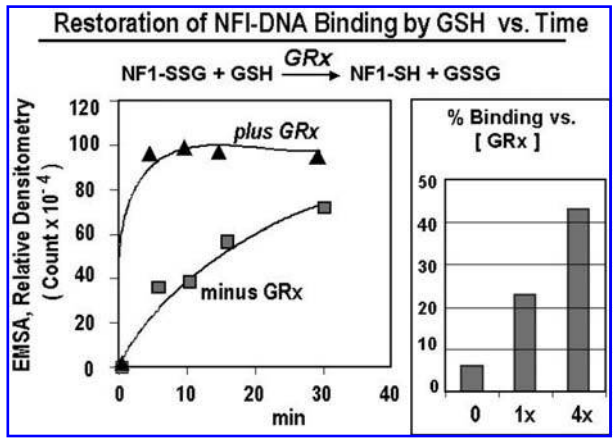
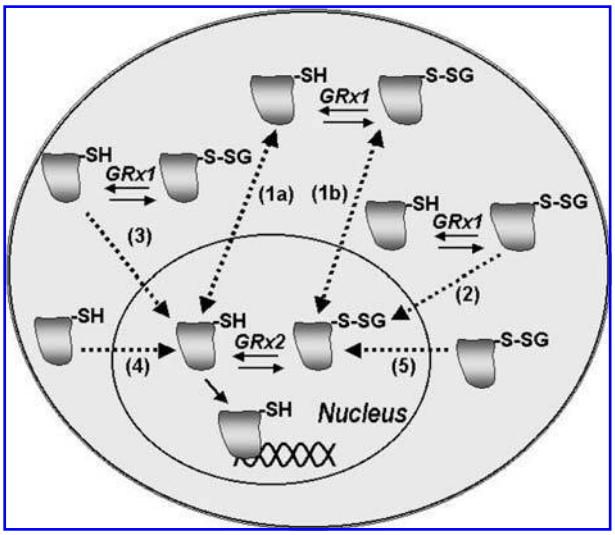


FIG. 5. Reactivation of NF1 by GRx. GRx restores DNA-binding activity to NF1 that had been oxidized by diamide in the presence of GSH. GRx catalyzes restoration of binding in a concentration-dependent fashion as illustrated. [For further discussion and experimental details, see Bandyopadhyay *et al.* (3).]

findings suggest an important role for GRx and intracellular GSH in the reduction of oxidant-sensitive cysteine residues in the NF1 family of transcription factors and potentially other site-specific DNA-binding proteins. However, as an example of reversible *S*-glutathionylation as a regulatory mechanism, the case for NF1 is not so strong as that for PTP1B and actin.

Collectively, the data provide circumstantial evidence for intracellular *S*-glutathionylation of NF1 in response to an oxidative stimulus that results in a change in function, *i.e.*, loss of DNA-binding activity, and GRx is implicated as the catalyst that restores the activity. There is as yet no direct evidence for NF1-SSG formation in cells in response to a physiological stimulus. GRx is an efficient catalyst of NF1 reactivation *in vitro*, but there is no direct evidence in this case for GRx action on NF1 in cells. Thus, the NF1 example at best fulfills only two of the criteria of Chart 1, so additional evidence is required to establish reversible *S*-glutathionylation as the specific mechanism of redox regulation of NF1 in cells, and this conclusion likewise applies to analogous transcription factors like NFkB and c-Jun.

Most studies of transcription factors use isolated nuclear extracts for gel shift assays and do not investigate regulation of translocation from the cytoplasm to the nucleus. Conceivably, *S*-glutathionylation could regulate NF1 translocation or DNA binding, or both. One study identified two nuclear localization sequences (NLS) that are conserved in all four NF1 genes (NF1-A, B, C, and X) and essential for translocation into the nucleus (54). These authors demonstrated that the presence of both NLS corresponded to nuclear localization, but an NF1 isoform with only one NLS was found in the nucleus and the cytoplasm. The two NLS are separated by conserved cysteines and at least 200 total amino acid residues (54). Besides possible conformational changes induced by glutathione modification of the cysteine residues, the negative charges of the glutathione moieties could attract positive charges on the basic residues of the NLS, thereby preventing them from performing their usual function in nuclear translocation. Scheme 3 pictures potential modes of regulation via



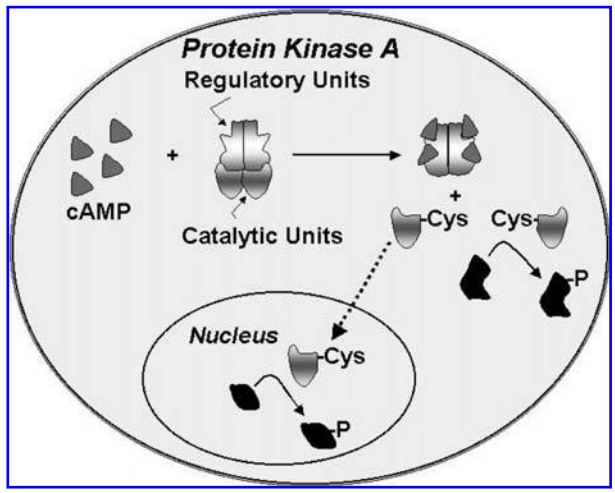
SCHEME 3. Regulation of transcription factors by GRx. This scheme pictures potential modes of regulation of transcription factors via reversible *S*-glutathionylation. Pathways 1, 2, and 3 depict regulation of nuclear translocation, whereas pathways 4 and 5 show no effect of *S*-glutathionylation on translocation; however, in both cases, the *S*-glutathionylated factor in the nucleus is expected to be unable to bind DNA. Thus, regulation of *S*-glutathionylation by cytoplasmic GRx1 could either promote (paths 1a and 3) or prevent nuclear translocation (paths 1b and 2). A double layer of regulation is also conceivable where GRx1 would regulate translocation and GRx2 (in the nucleus) would regulate DNA binding. [See text for further discussion.]

reversible *S*-glutathionylation. Pathways 1, 2, and 3 depict regulation of nuclear translocation, whereas pathways 4 and 5 show no effect of *S*-glutathionylation on translocation; however, in both cases, NF1-SSG in the nucleus is unable to bind DNA. Thus, regulation of *S*-glutathionylation by cytoplasmic GRx1 could either promote (paths 1a and 3) or prevent nuclear translocation (paths 1b and 2). A double layer of regulation is also conceivable where GRx1 would regulate NF1 translocation and GRx2 (in the nucleus) would regulate NF1 DNA binding. For continuity, this model is described for NF1, but it may apply broadly to transcription factors. In particular, regulation by this mechanism may be especially pertinent to transcription factors that are not constitutively active like NF1. In this regard, studies of oxidative regulation of the yeast activator protein-1 (yAP-1) transcription factor provide a good analogy for mammalian systems (23).

PKA and PKC

PKA. PKA, also known as cAMP-dependent protein kinase, is the primary mediator of the metabolic, morphological, and transcriptional effects of the second messenger cAMP, activated by hormones, growth factors, and neurotransmitters (51, 66). PKA is comprised of two regulatory and two catalytic subunits bound together as an inactive holoenzyme as depicted in Scheme 4. When cAMP binds to the regulatory subunits, the catalytic subunits dissociate and actively catalyze both reversible and irreversible cotranslational protein phosphorylation (66). The dissociated catalytic subunits reside in the cytosol where they phosphorylate cytosolic proteins, but they can also translocate to the nucleus and phosphorylate nuclear proteins such as CREB (66). The regulatory subunits control the dissociation and subsequent activation of PKA holoenzyme more so than cAMP.

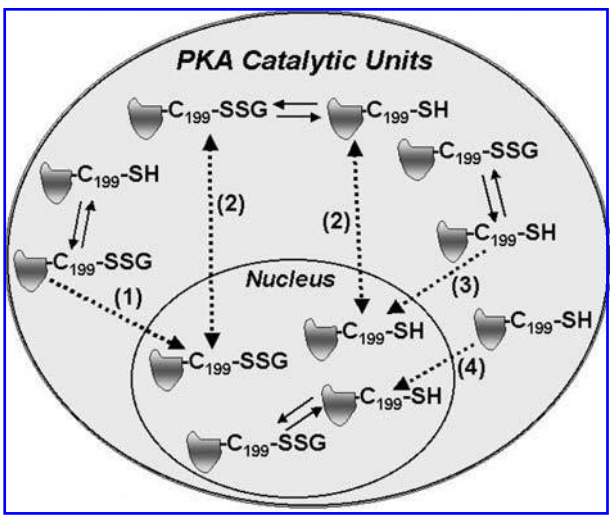
The catalytic subunits of PKA contain two cysteines, Cys199 and Cys343, that are exposed upon dissociation from the regulatory subunits (51). Of the two cysteine residues, Cys199 is conserved, located near the active site, and responsible for PKA inactivation upon sulfhydryl modifications (51). Consistent with a posttranslational regulatory role, mutation of Cys199 revealed that it is not essential for catalysis, but *S*-glutathionylation of Cys199 inhibited PKA activity (51). Upon cyanylation of the sulfhydryls, PKA exhibited only a 30% loss in activity, whereas conversion to glutathionyl mixed disulfides (PKA-SSG) by treatment with diamide plus GSH or Bio-GSH gave ~90% inactivation. The more complete inhibition by glutathione adduction suggests steric hindrance and/or electrostatic interference by the charges of the glutathionyl moiety that would not occur with the much smaller and neutral cyano moiety (51). Besides demonstrating PKA-SSG formation with the isolated catalytic subunits of the enzyme, Humphries *et al*. (51) showed that treatment of C3H 10T1/2 cells with diamide alone or diamide plus forskolin and butyryl-cAMP showed much greater inhibition of the enzyme activity in the latter case, where the enzyme was activated and the cysteine residues on the dissociated catalytic subunits were exposed. This finding reinforces the concept that modification of the cysteine residues intracellularly may represent an actual regulatory event; however, the use of diamide rather than a physiological redox stimulus limits the impact of this observation. The authors demonstrated reversibility of the diamide-driven glutathiony-



SCHEME 4. Regulation of PKA by cAMP. PKA is comprised of two regulatory and two catalytic subunits in an inactive holoenzyme as depicted in this scheme. When cAMP binds, the catalytic subunits dissociate and catalyze both reversible and irreversible cotranslational protein phosphorylation. The dissociated catalytic subunits reside in the cytosol and phosphorylate cytosolic proteins, but they can also translocate to the nucleus and phosphorylate nuclear proteins. [For further discussion, see text and Kopperud *et al.* (66).]

lation of PKA with DTT and with GRx, with GRx displaying selective deglutathionylation of Cys199. Remarkably, they also reported that GRx was able to reactivate the enzyme that had been adducted with Bio-GSH (PKA-SSG-Biotin), although the time course was not reported. This result is surprising in light of the stringent specificity of GRx for the glutathionyl moiety as described above (47, 79, 117), and it suggests that GRx may be able to catalyze the deglutathionylation despite the presence of the bulky biotin adduct on the glutathionyl moiety, but the catalytic efficiency relative to removal of the normal glutathionyl moiety is likely to be low.

In summary, regulation of PKA by reversible *S*-glutathionylation fulfills at best only two of the criteria of Chart 1 (above); however, the prospect of documenting this mechanism as a component of the multi-staged regulation of the PKA enzyme in future studies is a worthy venture. It is fascinating to contemplate the many layers of regulation of PKA, including intersubunit interactions, covalent modification by glutathione, subcellular localization by A-kinase anchoring proteins, and translocation to the nucleus. As with the consideration of NF1, it is conceivable that *S*-glutathionylation may affect activity and/or nuclear translocation of the PKA catalytic subunits. It would be interesting to discover whether PKA inhibition upon *S*-glutathionylation occurs in the cytoplasm as a prerequisite for nuclear translocation, occurs in the cytoplasm preventing nuclear translocation and/or subcellular anchoring, or occurs in the nucleus after translocation from the cytoplasm. These possibilities are depicted in Scheme 5.



SCHEME 5. Model for regulation of PKA by reversible *S*-glutathionylation. It is conceivable that *S*-glutathionylation may affect activity and/or nuclear translocation of the PKA catalytic subunits. It remains to be discovered whether PKA inhibition upon *S*-glutathionylation occurs in the cytoplasm as a prerequisite for nuclear translocation, occurs in the cytoplasm preventing nuclear translocation and/or subcellular anchoring, or occurs in the nucleus after translocation from the cytoplasm. These possibilities are depicted in this scheme. [See text and Humphries *et al.* (51) for further details.]

PKC. The PKC family includes 10 or more serine/threonine kinase isozymes primarily involved in cell growth, differentiation, stress response, and death (46, 113). The isozymes are categorized into three groups based on their regulatory domains and cofactor dependence: classical calcium-dependent or cPKC (α, β_1, β_2, γ), novel calcium-independent or nPKC (ϵ, δ, θ, η) and atypical or aPKC (ζ, ι) (67, 113). Upon activation, PKC translocates from the cytoplasm to the plasma membrane (46, 67). Three phosphorylation sites, subcellular localization, and *S*-glutathionylation are proposed to be key modes of PKC regulation (69, 113). The PKC isozymes have 16–28 cysteine residues (18) that are distributed throughout the protein sequence (46). Chemical modifications of the cysteine within the catalytic domain inactivate PKC analogous to those that inactivate the catalytic domains of PKA (46). In contrast, modifications of the cysteines within the regulatory domain activate PKC (46). The regulatory domain contains a total of 12 cysteine residues that, when in the appropriate conformation, bind a total of four zinc metal ions generating autoinhibition (46). Conversely, the cysteines within the catalytic domain do not coordinate metals and readily react with sulfhydryl modifying agents (46).

PKC-α, in particular, contains 20 cysteine residues. Treatment of isolated PKC-α with diamide and GSH leads to *S*-glutathionylation (documented by [^{35}S]GSH incorporation) and concomitant inactivation. In addition, diamide treatment of NIH3T3 cells (pretreated with cycloheximide and L-[^{35}S]cysteine) led to inactivation and *S*-glutathionylation of the PKC, as documented by analysis of the enzyme isolated by immunoprecipitation (113); however, it is not clear how many cysteine residues were glutathionylated. Besides DTT, GRx was reported to reactivate the PKC-SSG, but no detail was given, so the efficiency and site selectivity of the GRx deglutathionylation cannot be evaluated. These authors indicated their plan to make cys→ala mutations in order to identify which *S*-glutathionylated cysteine residue(s) is responsible for enzyme inactivation.

Humphries *et al.* (51) have predicted that *S*-glutathionylation of Cys499 of PKCα is responsible for inactivation of that enzyme, *i.e.*, the homologue of Cys199 of PKA. Six other PKC isozymes (cPKCβ_1, cPKCβ_2, cPKCγ, nPKCδ, nPKCϵ, and aPKCζ, as well as protein kinase D, have been shown to be inactivated by *S*-glutathionylation *in vitro,* with nPKCδ displaying the most resistance to *S*-glutathionylation (17). PKCδ and PKCϵ additionally have been shown to be activated and inactivated, respectively, by *S*-cysteinylation *in vitro* and in COS7 cells transfected with PKC and treated with cysteine dimethyl ester (18, 19). PKCγ also was shown to be inactivated by *S*-cysteinylation *in vitro* (18). Although identifying the various PKC isozymes as potential targets of redox regulation via *S*-glutathionylation, the studies to date do not go very far toward satisfying the criteria of Chart 1 (above).

By changing its activity, *S*-glutathionylation of PKCβ1 would likely cross-regulate the actin and NFκB pathways (1), as alluded to above. Besides altering PKC activity, *S*-glutathionylation potentially may also regulate translocation of PKC to the plasma membrane, where it is typically found to be in its active state (46). Modulation of PKC migration to the plasma membrane in response to a change in *S*-glutathionylation state would be reminiscent of actin transloca-

tion to the cell periphery upon de-glutathionylation, as discussed above. Understanding the factors that regulate PKC is essential to characterizing the signaling cascades where it plays a role, such as the NFκB pathway, which depends on NFκB translocation from the cytoplasm to the nucleus for its transcriptional activity (62).

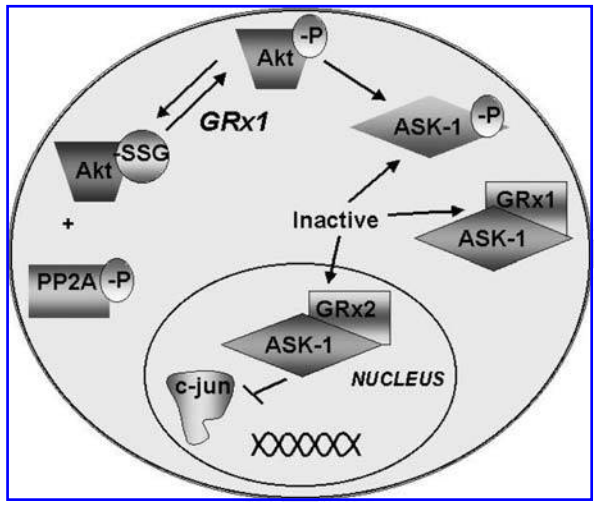
ASK-1 and Akt

ASK-1 and Akt are kinases that are implicated in regulation of apoptosis, and they interact with each other. As described below, their activities are modulated directly or indirectly by *S*-glutathionylation events and association with GRx.

ASK-1 is a serine, threonine protein kinase that regulates apoptosis by activating c-Jun N-terminal kinase (JNK) and p38 MAP kinase pathways (92). ASK-1 can be positively regulated by phosphorylation of Ser845 and negatively regulated by phosphorylation of Ser83 and Ser967 (45, 60, 82). Dephosphorylation of Ser845 and subsequent inactivation are mediated by protein phosphatase 5, which forms a complex with ASK-1 (82). When Ser967 is phosphorylated, ASK-1 binds to 14–3-3, a phosphoserine/phosphothreonine binding molecule (45). Upon dephosphorylation of Ser967, ASK-1 dissociates from 14–3-3, and ASK-1 activity is enhanced (45). ASK-1 activity is decreased by phosphorylation of Ser83 mediated by Akt, also known as protein kinase B and related to A and C protein kinase (RAC-PK) (26, 60). Akt also phosphorylates Bad on Ser136, which is necessary for Bad-14–3-3 complex formation in a cell survival pathway (26). Akt deactivation has been associated with multiple cell death pathways, suggesting a protective role for activated Akt (75). Phosphorylation of Thr308 and Ser473 leads to Akt activation (83).

S-glutathionylation is reported to regulate the serine-threonine phosphatase protein, phosphatase 2A (PP2A), that inactivates Akt by dephosphorylation of two different residues (83, 88). In addition, overexpression of GRx in H9c2 cells was reported to protect Akt from H_2O_2-induced oxidation and prevent its interaction with PP2A (83), suggesting that Akt itself or one of its partners is *S*-glutathionylated under oxidative stress and deglutathionylated by GRx. However, yeast GRx was used in this study, which complicates the interpretation because this form of GRx is reported to display H_2O_2-scavenging glutathione peroxidase activity.

Besides posttranslational modifications, ASK-1 activity also depends on direct protein–protein interactions. Thus, ASK-1 is inhibited by a redox-regulated binding of its N-terminus to TRx in human 293 embryonal kidney cells (92). Analogously, GRx1 binds to ASK-1 in MCF-7/ADR cells, but this redox-dependent inhibitory interaction occurs at the C-terminus of ASK-1 (98). Thus, oxidative conditions disrupt both TRx and GRx complexes with ASK-1. The two ASK-1 interactions are distinct in that TRx dissociation was GSH-independent, whereas GRx dissociation was dependent on the concentration of GSH in DU 145 human prostate adenocarcinoma cells (97), suggesting some connection to the glutathionyl specificity and catalytic activity of GRx (79). By directly binding to ASK-1, GRx prevents ASK-1 activation and subsequent activation of downstream pathways such as JNK and p38, which regulate translocation of transcription factors from the cytoplasm to the nucleus analogous to the nuclear localization of ASK-1 that occurs in response to its dissociation from ALG-2 (apoptosis-linked gene-2) (53). ALG-2 binds ASK-1, preventing nuclear translocation and c-Jun activation (53). Therefore, it may be surmised that an oxidative signal causing dissociation of the ASK-1-GRx complex would facilitate activation of JNK and p38, which activate downstream targets and subsequent gene expression. For example, activated JNK phosphorylates, activates, and induces nuclear translocation for DNA transcription of c-Jun, which itself has been found to be *S*-glutathionylated (64). Thus, *S*-glutathionylation and its reversal by GRx(1 or 2), as well as complex formation with GRx(1 or 2), may have multiple regulatory roles along the Akt and ASK-1 pathways, as illustrated in Scheme 6; however, much additional characterization is necessary.



SCHEME 6. Alternative roles of GRx in regulation of cell signaling. *S*-Glutathionylation and its reversal by GRx(1 or 2), as well as complex formation with GRx(1 or 2), may have multiple regulatory roles along the Akt and ASK-1 pathways as illustrated in this scheme. [See text for further discussion.]

V. CONCLUSIONS AND FUTURE DIRECTIONS

Much circumstantial evidence has accrued to support the concept of reversible *S*-glutathionylation as a cellular regulatory mechanism akin to reversible *O*-phosphorylation. *S*-glutathionylation likely represents an interactive cross-modulatory mechanism that fine-tunes the net phosphorylation cascade of events for transducing and integrating signals that control cellular functions in response to external stimuli. Examination of current data for specific regulatory proteins that reversibly form protein-SSG intermediates within cells in response to physiologically relevant signals identifies very few examples that establish *S*-glutathionylation as a *bone fide* cellular regulatory mechanism. Yet there is much warranted enthusiasm for continuing the study of candidate proteins *in vivo* that are subject to redox regulation. The abundance of GSH in cells and the ready conversion of sulfenic acids and

S-nitroso derivatives to *S*-glutathione mixed disulfides supports the notion that *S*-glutathionylation is likely to be the preponderant mode of redox signal transduction. The GRxs have emerged as the primary catalysts of de-glutathionylation, whereas identification of the enzyme(s) that catalyze formation of protein-SSG intermediates represents a frontier objective for characterization of this evolving mechanism of cellular regulation. The final section is a future-directed discussion of the cross-talk between *O*-phosphorylation and *S*-glutathionylation events using regulation of NFκB signaling as the focal point.

NFkB as a model for future integration of signaling mechanisms

NFκB and its upstream signaling mediators are regulated by phosphorylation and have been shown to have altered DNA-binding affinity upon *S*-glutathionylation *in vitro* (87). Analogous to the multiple intermediate points of phosphorylation, at least two specific points of potential *S*-glutathionylation can be recognized along the NFκB pathway, namely, NFκB itself and the IκB kinases (IKKs) that facilitate activation of NFκB by promoting IκB dissociation. Both NFκB and IKK have been shown to have critical cysteines that are susceptible to *S*-glutathionylation (87, 91). NFκB is a redox-sensitive transcription factor comprised of homo- or heterodimers of five different Rel family subunits. Three of the subunits, Rel A (p65), Rel B, and c-Rel, activate transcription, whereas the other two, p50 and p52, possess DNA-binding properties (96). NFκB dimers comprised of p50 and p65 are the most common combination of subunits (37). Inactive NFκB rests in the cytoplasm bound and sequestered by inhibitory proteins (IκBs), but it can be activated by cell signals such as inflammatory cytokines, oxidants, and PKC (30, 81). The cell signaling events that lead to NFκB activation include activation of IKK that phosphorylates two specific serine residues at the N-terminus of the IκBs, which primes the protein for degradation (30, 37).

The activation of IκBs is directly related to the activity of IKKs, which are a primary target for regulation by *S*-glutathionylation (87). IKKs have been proposed to phosphorylate IκBs, as well as the p65 subunit of NFκB itself (62, 96). Nuclear translocation of NFκB has been shown to correspond to IKK activation (48, 62, 96). IKK is an upstream mediator in the NFκB pathway and has a critical cysteine in its activation loop, Cys179 (91). *S*-Glutathionylation of IKK could present a likely means of enzymatic regulation similar to that of the kinases (PKA, PKC, and ASK-1) discussed above. The scenario described here is only a sampling of the network of interactions among phosphorylation and glutathionylation events that remain to be described and understood.

ABBREVIATIONS

aPKC, atypical protein kinase C; ASK-1, apoptosis signaling kinase-1; Bio-Cys, biotinylated cysteine; Bio-GEE, biotinylated glutathione ethyl ester; Bio-GSH, biotinylated glutathione; Bio-NEM, biotinylated *N*-ethylmaleimide; cAMP, cyclic AMP; cPKC, classical calcium-dependent protein kinase C; CREB, cAMP response element binding protein; DTT, dithiothreitol; EGF, epidermal growth factor; FGF, fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRx, glutaredoxin; GSH, reduced glutathione; GS-NO, *S*-nitrosoglutathione; GSSG, glutathione disulfide; H_2O_2, hydrogen peroxide; IAF, 5-iodoacetamidofluorescein; IAIT, *N*-iodoacetyl-[^{125}I]iodotyrosine; IκB, inhibitor of nuclear factor-κB; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated kinase; NEM, *N*-ethylmaleimide; NF1, nuclear factor 1; NFκB, nuclear factor-κB; NLS, nuclear localization sequence(s); nPKC, novel calcium-independent protein kinase C; PKA, protein kinase A; PKC, protein kinase C; PP2A, protein phosphatase 2A; protein-SSG, protein glutathione mixed disulfide; PTP, protein tyrosine phosphatase; PTP1B, protein tyrosine phosphatase-1B; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TNF-α, tumor necrosis factor-α; TRx, thioredoxin.

NOTE ADDED IN PROOF

After this review was prepared, a study was reported that provides another excellent example of regulation by *S*-glutathionylation [Adachi T, Pimentel DR, Heibeck T, Hou X, Lee YJ, Jiang B, Ido Y, and Cohen RA. Glutathionylation of Ras mediates redox-sensitive signaling by angiotensin II in vascular smooth muscle cells. *J Biol Chem* 279: 29857–29862, 2004]. Angiotensin II (AII) treatment of vascular smooth muscle cells rapidly induced *S*-glutathionylation and activation of Ras, detected and isolated *via* Bio-GEE and streptavidin-sepharose. Overexpression of GRx1 decreased Ras-SSG, and concomitantly decreased the AII-induced phosphorylation of downstream effectors p38 and Akt, and stimulation of protein synthesis. These observations document Ras-*S*-glutathionylation as a regulatory event in signal transduction from the angiotensin receptor, fulfilling the criteria of Chart 1 (above). Thus, actin, PTP1B, and Ras currently represent the best characterized signaling intermediates regulated by reversible *S*-glutathionylation involving glutaredoxin. Remarkably these three proteins display three different responses to physiological stimuli that initiate signal transduction via generation of intracellular ROS.

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