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Causes and Consequences of Cysteine *S*-Glutathionylation*

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Post-translational *S*-glutathionylation occurs through the reversible addition of a proximal donor of glutathione to thiolate anions of cysteines in target proteins, where the modification alters molecular mass, charge, and structure/function and/or prevents degradation from sulfhydryl overoxidation or proteolysis. Catalysis of both the forward (glutathione *S*-transferase P) and reverse (glutaredoxin) reactions creates a functional cycle that can also regulate certain protein functional clusters, including those involved in redox-dependent cell signaling events. For translational application, *S*-glutathionylated serum proteins may be useful as biomarkers in individuals (who may also have polymorphic expression of glutathione *S*-transferase P) exposed to agents that cause oxidative or nitrosative stress.

S-Glutathionylation targets cysteines in a basic environment (low pK_a) perhaps in close three-dimensional proximity to Arg, His, or Lys residues. Reports of reversible *S*-glutathionylation emerged as early as 1985 (1), but in concert with understanding the importance of reactive oxygen/nitrogen species (ROS/RNS)[2] as second messengers, publications have increased substantially over the past 10 years. ROS/RNS signaling operates through a set of post-translational protein modifications that are discrete, site-specific, and reversible. Certain proteins undergo reversible chemical changes in response to altered localized redox potential. Among the most susceptible redox-sensitive targets are thiol (–SH) groups on cysteines. Signaling events are facilitated through redox-active proteins when one or more cysteines can exist as reactive thiolate anions. These cysteines are more nucleophilic and become susceptible to attack by GSH.

Oxygen-based metabolism is arguably the most efficient and evolved method for producing energy from nutrients, but ROS as byproducts of ATP generation provide physiological challenges. There are >200,000 cysteines encoded by the human genome, making cysteine one of the least commonly used amino acids (2). This sparing usage does not imply restricted functionality but instead indicates evolutionary importance particularly because organism cysteine content correlates with degree of biological complexity (3). Sulfur has unique characteristics (variable valence, nucleophilicity, and capacity to exist in different oxidation states) that make it intrinsically useful in a biologically oxidative environment. The major non-protein source of cysteine in cells is GSH (γ-glutamylcysteinylglycine), where the sulfhydryl can be deprotonated to a thiolate anion. Reported pK_a values for the thiol group of GSH range from 8.7 (4) to 9.25 (5), with variation contingent upon the measurement methodology. Balance between oxidation and reduction reactions plays an essential role in numerous signaling cascades, including those associated with proliferation, inflammatory responses, apoptosis, and senescence. Post-translational modifications of cysteines can directly influence these processes. The flexibility of the thiol group of cysteine predicates that a number of post-translational modifications can occur (Table 1). As a response to ROS/RNS, *S*-glutathionylation is an established modification. The basal levels of *S*-glutathionylated proteins in resting cells are at least an order of magnitude lower than those in cells under oxidative stress (6). Quantitative analysis of the relationship between cysteine modifications and the corresponding functional changes in various cellular contexts is needed to define the frequency of *S*-glutathionylation events *versus* other cysteinyl modifications. Bioinformatic approaches may prove useful in predicting the susceptibility of individual cysteines to *S*-glutathionylation (7). Furthermore, *S*-nitrosylation may precede and initiate *S*-glutathionylation (8–10).

Thiol Modifications Caused by ROS

ROS include oxygen-centered radicals (singlet oxygen ($^1O_2$), hydroxyl radical ($^{\bullet}OH$), superoxide anion radical ($O_2^{\bullet-}$, SO), and peroxyl radical (R-$O_2^{\bullet-}$)) as well as hydrogen (and other organic) peroxide (H_2O_2, ROOH) and ozone (O_3). Protein cysteines can occur as surface residues generally exposed to an aqueous environment, as is predominant in mitochondria (11), or buried inside globular domains. Either could be catalytically or structurally significant.

Regardless of their origin, neither $^1O_2$ nor $^{\bullet}OH$ can be considered as a specific protein thiol oxidant. Contributory factors to this include their short half-life ($\sim 10^{-9}$ s) (12) and high nonspecific reactivities with nearly diffusion-controlled rate constants (13). SO can oxidize thiols to thiyl radicals at a rate constant of $\sim 10^3$ M^{-1} s^{-1} at pH 7.4 (14). Because the rate constant for cytosolic and mitochondrial superoxide dismutases is $\sim 10^7$–10^9 M^{-1} s^{-1} (15) and that for spontaneous dismutation of SO is $\sim 10^5$ M^{-1} s^{-1} (16), protein thiol oxidation by SO is too

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[2] The abbreviations used are: ROS, reactive oxygen species; RNS, reactive nitrogen species; GSTP, glutathione *S*-transferase P; Grx, glutaredoxin; Sec, selenocysteine; ER, endoplasmic reticulum; HSC, hematopoietic stem cell; HPC, hematopoietic progenitor cell.

TABLE 1
Possible post-translational modifications of protein-associated cysteine residues

Group added to Cys residue	Notes
Thiol, disulfide, thiolate, sulfenic, sulfinic, or sulfonic acids	These occur following different degrees of ROS exposure, causing different extents of oxidation.
S-Cysteinylation	This occurs in ROS, where a disulfide bond converts L-Cys to *S*- (L-cysteinyl)-L-Cys. Donors can be cysteine or GSSG.
S-Nitrosylation	This involves NO-mediated formation of *S*-nitrosothiol and is reversible and sometimes short-lived. Consequences are important in cell signaling events.
S-Glutathionylation	This is a consequence of GS^- addition, with both protective and signaling consequences.
S-Sulfhydration	This is a physiological post-translational modification caused by hydrogen sulfide, with subsequent effects on cell signaling pathways.
S-Isoprenylation, *S*-farnesylation, *S*-geranylgeranylation, and *S*-palmitoylation	These involve lipidation through addition of hydrophobic residues. Frequently, the affected protein has modified affinity for other proteins. Although particularly relevant in cell membranes, a number of enzymes are influenced by the process.

slow to be significant. Both spontaneous and catalyzed dismutations of SO result in an accumulation of H_2O_2. Generally, the intracellular levels of H_2O_2 are controlled by catalase (an enzyme with a high turnover rate) (17), GSH peroxidases 1–4 (18), and peroxiredoxins (19). Site-specifically, H_2O_2 levels can be elevated enough to cause thiol oxidation. Thus, H_2O_2 and ROOH specifically oxidize thiols through a two-electron nucleophilic substitution reaction (13). This reaction requires dissociation (deprotonation) of the thiol to a thiolate anion (RS^-) to reach the required nucleophilicity (13). The pK_a of protein cysteines approximates 8.5 (4). Thus, only a small proportion of protein thiols are deprotonated at physiological pH (pH 7.0–7.4). Cysteines within a basic environment (*e.g.* three-dimensionally surrounded by Arg, Lys, or His residues) have higher reaction rates with H_2O_2 primarily as a consequence of greater deprotonation. One result of this oxidation is formation of cysteine sulfenic acid, which is highly reactive with the GSH sulfhydryl and can facilitate protein *S*-glutathionylation (20). Fig. 1*A* provides an outline of some of these reactions.

GSH is in most cells at millimolar levels (21). The reasons for the nonspecific reactivity of GSH with $^1O_2$ and $^{\bullet}OH$ are the same as for protein thiols (see above). Reaction of GSH with SO usually generates GSSG and an intermediate thiyl radical ($GS^{\bullet}$) and thiolate anion (GS^-) (22). Because of the high concentrations of GSH and the instability of GSH sulfenate as an intermediate, the reaction of GSH with H_2O_2 (ROOH) also generates GSSG (22). GSH reductase reduces intracellular GSSG to GSH using NADPH as a source of electrons (23). A high cellular content of GSH could in principle result in site-specific accumulation of GSSG and thiol-disulfide exchange-driven *S*-glutathionylation of proteins.

Thiol Modification by RNS

RNS include moieties such as nitric oxide (NO) and NO-derived compounds, nitroxyl anion (NO^-, HNO), nitrosonium cation (NO^+), higher oxides of nitrogen (N_2O, NO_2, N_2O_3), peroxynitrite ($ONOO^-$/ONOOH), *S*-nitrosothiols (RSNO), and dinitrosyl iron complexes ($(RS^-)_2Fe^+(NO^+)_2 \ldots (^-SR)_2)^-$) (24).

NO is a reactive molecule generated intracellularly by NOS that may nitrosylate protein thiols directly, through reaction with molecular oxygen (generating N_2O_3 with high affinity for thiols) (25) or with SO radicals (generating peroxynitrite) (26). Generally, the half-lives of nitrosylated cysteines are short as a

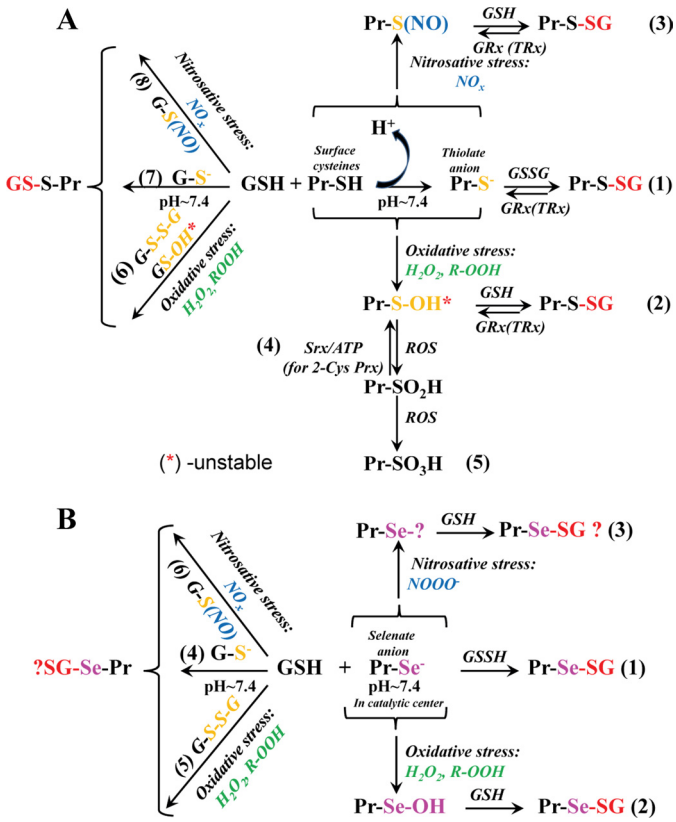

FIGURE 1. **Chemical basis for the biological *S*-glutathionylation of regular Cys (*A*) and Sec (*B*).** *A*, surface protein cysteines at physiological pH are not reactive because of high pK_a values. In a basic microenvironment, the thiol deprotonates to a thiolate anion, which attacks the sulfhydryl of intracellular GSSG, with the subsequent formation of disulfide (*pathway 1*). Under oxidative (*pathway 2*) or nitrosative (*pathway 3*) stress, a surface protein (*Pr*) sulfhydryl becomes activated through cysteine sulfenate or nitro-*S*-cysteine formation and then undergoes *S*-glutathionylation. Deglutathionylation of glutathionylated cysteines is predominately catalyzed by Grx. Site-specifically, thioredoxin (*TRx*) can also catalyze deglutathionylation. Sulfiredoxin (*Srx*) catalyzes the reduction of a protein cysteine sulfinic acid into sulfenate for 2-Cys peroxiredoxins (*Prx*; *pathway 4*). Generally, overoxidation of protein cysteines is terminal (*pathway 5*). Similar to protein thiols, the cysteine of GSH can be activated through *pathways 6–8*, resulting in protein *S*-glutathionylation. The relative abundance of intracellular GSH influences the probability and specificity of particular reactions (see text). *B*, for proteins containing Sec (GSH peroxidases, etc.), its low pK_a results in the formation of a selenate anion at physiological pH, permanently activated (*pathway 1*). *S*-Glutathionylation of protein Sec residues is affected by ROS/RNS (*pathways 2* and *3*), but the mechanism of the latter is unclear. *S*-Glutathionylation of Sec could be further accelerated by activation of GSH (*pathways 4–6*), but the mechanism of such is not yet defined.

consequence of spontaneous denitrosylation by excess GSH (generating GSNO). However, for a few proteins, *S*-nitrosylation may be stabilized by conformation-mediated inaccessibility for GSH (27). Glutathione *S*-transferase P (GSTP) in the presence of excess GSH can be *S*-glutathionylated, providing another (albeit indirect) pathway of NO signaling. The reaction of peroxynitrite with thiols can generate thiyl radicals or sulfenates (13), with the implication that the forward reaction of *S*-glutathionylation might be independent of GSH/GSSG ratios.

A primary role for glutaredoxin (Grx) in deglutathionylation is premised on the nucleophilic attack of the cysteine thiolate anion on a protein-GSH mixed disulfide (28). There are reports that Grx can catalyze GSSG-dependent *S*-glutathionylation of proteins such as actin, GAPDH, and protein-tyrosine phosphatase 1B by an alternative mechanism involving $^{\bullet}$OH-mediated GSH thiol radical generation under hypoxia (29). Considering the high reactivity and nonspecificity of $^{\bullet}$OH-mediated reactions, the overall contribution of this mechanism of catalysis remains to be established.

In the presence of O_2, NO may react with the sulfhydryl of GSH to produce (likely through intermediate(s) of higher nitrogen oxides) GSNO, which can serve as a physiological scavenger of NO_x species (30). GSNO can react with GSH, yielding GSSG, nitrite, nitrous oxide, and ammonia (31), products that may contribute to protein nitrosylation. Protein thiol transnitrosylation by GSNO (28) is regulated by expression of GSNO reductase (32). High intracellular GSH concentrations can shift the equilibrium of the reaction with NO to GSSG, but during nitrosative stress, certain cell compartments can harbor high levels of GSNO. Under such conditions, GSNO may contribute to protein nitrosylation and/or *S*-glutathionylation (9, 10). The specificity of GSNO-mediated post-translational modifications depends on the microenvironment of the target cysteine within protein tertiary and quaternary structures.

Can Selenium Substitute for Sulfur?

Selenium and sulfur are in the same group in the periodic table and share common properties, including participation in oxidation/reduction reactions. Selenium is found in the form of selenocysteine (Sec; the 21st amino acid), coded by the UGA codon in 25 proteins (33). From an evolutionary standpoint, recoding the UGA stop codon into the Sec sense codon requires commitment of biological energy, implying that it fulfills some critical biological role that sulfur-containing cysteines do not. Selenol groups are substantially more acidic than thiols, with pK_a values of ~5.3 (34). At physiological pH, selenol is completely deprotonated and exists as a selenate anion (RSe^-). Selenyl radicals are less oxidizing than thiyl radicals and are more easily produced (35). Generally, Sec is less stable than Cys (22), and native selenoproteins do not necessarily have higher activities than native sulfur proteins (36). Selenols (as selenate anions) are better nucleophiles than thiols and become oxidized by H_2O_2 (ROOH) to selenic acid (RSeOH). Selenic acid is unstable and reacts with GSH, forming a selenenyl sulfide adduct (glutathionylated selenenyl). A second molecule of GSH reduces this selenenyl sulfide bond to selenol and generates GSSG (37). These reactions occur in the catalytic center of GSH peroxidases and may require a more hydrophobic environment. Oxidations of surface protein selenols and their subsequent reactivity with GSH have not been demonstrated, and overoxidation of Sec to seleninic or selenonic acid has not been reported (38).

GSH peroxidase can be inactivated by peroxynitrite (39), and stop-flow experiments have monitored its reaction with selenate anions (*E*-Se^-) of Sec in the enzyme active site. This reaction can transform the selenate anion into a form of selenium that is difficult to reduce back to selenol (39). This reaction occurs in the specific hydrophobic environment of the enzyme catalytic center. To date, there are no reports that RNS may mediate the reactivity of Sec residues with GSH. Fig. 1*B* summarizes some of these reactions. In summary, selenium and sulfur have not evolved to perform identical antioxidant functions within the cell.

S-Glutathionylation as a Cycle

The forward reaction of *S*-glutathionylation can be either spontaneous or catalyzed by GSTP. At physiological pH, GSTP binds GSH and lowers the pK_a of the thiol, producing a thiolate anion (GS^-) at the active site (40). This catalyzes the forward reaction, a specific example of which is provided by the reactivation of peroxiredoxin-6 (41). Reversal of *S*-glutathionylation may be impacted by enzymes such as thioredoxin (42), Grx (43), and sulfiredoxin (44). Although studies have confirmed a role for Grx in protein deglutathionylation (45), Grx may also facilitate GS radical recombination with a protein thiyl radical and catalyze *S*-glutathionylation via stabilization of the $GS^{\bullet}$ thiyl radical as an enzyme disulfide anion radical intermediate (Grx1-$SSG^{\bullet-}$) (46). Reversal of *S*-glutathionylation is contingent upon the extent and duration of the initial stress, removal of which usually permits deglutathionylation to follow a half-life of ~2–3 h (47). Relatively high levels of ROS would give precedence to the protective function, masking critical thiols from further irreversible oxidation. Whereas sulfinates or sulfonates frequently lead to proteolysis, *S*-glutathionylated residues can maintain dormancy during stress.

Relative to the proteome, the number of proteins susceptible to *S*-glutathionylation is not large. Proteomic techniques reveal a high number of putative *S*-glutathionylated proteins in unicellular organisms such as *Plasmodium falciparum* and *Chlamydomonas reinhardtii* (48, 49). A literature review reveals some clustering of function and includes enzymes with catalytically important cysteines (in particular, those involved with protein folding and stability, NO regulation, and redox homeostasis); cytoskeletal proteins; signaling proteins (particularly kinases and phosphatases); transcription factors; Ras proteins; protein folding and degradation; ion channels, calcium pumps, and binding proteins (involved in calcium homeostasis); and energy metabolism and glycolysis. Within these categories, there are some notable examples of functional alterations associated with the modification: cytoskeletal restructuring during cell growth or motility is impacted by *S*-glutathionylation, particularly of actin (50). Some studies report inhibition of enzymatic activity/protein function by *S*-glutathionylation, *e.g.* GAPDH (51), eNOS (52, 53), the heteromeric Kir4.1-Kir5.1 channel (54), low molecular weight tyrosine phosphatase (55),

MAPK phosphatase 1 (56), mitochondrial thymidine kinase 2 (57), Na,K-ATPase (58), and protein-disulfide isomerase (59). The position of cysteine is important in determining the impact of the modification. For instance, carbonic anhydrase III can be inhibited or activated depending on the cysteine involved (60), whereas the inositol 1,4,5-triphosphate receptor (61) and Rac2 (62) are each activated by *S*-glutathionylation. The canonical phosphorylation/dephosphorylation pathways are cyclical, flexibly controlling signaling events as a response to external or internal stimuli. A significant number of kinases and phosphatases are subject to secondary regulation by *S*-glutathionylation, providing secondary control of critical pathways following stimuli that cause homeostatic changes in ROS/RNS. Additional detailed examples are discussed in recent reviews (63, 64).

One emerging example merits emphasis. Fas (CD95, Apo-1) is a constitutively expressed member of the TNF family of death receptors with roles in apoptosis signaling. Additional regulatory roles of Fas, including immune cell activation and proliferation, have been identified (65). Fas is a substrate for *S*-glutathionylation (Fas-SSG) at Cys-294, sustained via caspase-dependent degradation of Grx1 (66). Fas-SSG enhances Fas recruitment into lipid rafts and promotes FasL binding, the formation of death-inducing signaling complexes, and caspase activation, resulting in amplification of cell death pathways (66). Distinct subcellular localization of Fas exists, with oxidative conditions causing the majority of Fas-SSG to be found in the endoplasmic reticulum (ER). Moreover, stimulation with FasL causes protein/protein interactions between Fas, ERp57 (a member of the protein-disulfide isomerase family), and GSTP. Genetic or pharmacologic diminution of ERp57 and GSTP1 decreases FasL-induced *S*-glutathionylation of Fas, producing lowered death-inducing signaling complex formation and enhanced cell survival. Increased expression of ERp57 and GSTP1 enhances the kinetics of translocation of Fas-SSG from the ER to the cytosol/plasma membrane, enhances caspase activation, and promotes cell death. As a disease correlate, interactions between Fas, ERp57, and GSTP1 and levels of Fas-SSG are increased in murine pulmonary fibrosis, which depends on functional Fas, implying that oxidative processing of Fas and its subsequent *S*-glutathionylation may provide an important ligand regulatory switch to control the apoptotic signal (67). Furthermore, *S*-glutathionylation of the GTPase Ras does not affect Ras activity unless it is modified by GSH through a radical-mediated mechanism, which promotes activation through nucleotide dissociation and GTP binding. Binding of GSSG through disulfide exchange or via Ras oxidation by a non-radical oxidant does not impact Ras activation (68).

Translational Applications for Protein *S*-Glutathionylation

Human response to ROS/RNS is influenced by numerous variables. For example, GSTP polymorphisms arise from nucleotide transitions that change codon 105 from Ile to Val and codon 114 from Ala to Val, generating four possible GSTP1 alleles: wild-type GSTP1-1A (Ile-105/Ala-114), GSTP1-1B (Val-105/Ala-114), GSTP1-1C (Val-105/Val-114), and GSTP1-1D (Ile-105/Val-114). Population frequencies of 0.65 (GSTP1-1A), 0.262 (GSTP1-1B), and 0.068 (GSTP1-1C) occur in Caucasians (69). The I105V and A114B substitutions do not alter GSH binding affinity but cause steric changes at the substrate-binding site of the enzyme (70). What impact GSTP polymorphisms may have in establishing risk factors for response to ROS/RNS is not clear. One example is provided by the hierarchical effectiveness of polymorphic variants of GSTP in regulating the peroxidase activity of Prdx6 (71). Prdx6 is a dual-functioning enzyme that detoxifies lipid peroxides particularly in biological membranes with a peroxidase function activated by GSTP. Activation of Prdx6 depends on its heterodimerization and subsequent *S*-glutathionylation by GSH-loaded GSTP (41). The catalytic Cys-47 residue is buried in the hydrophobic globular core region of Prdx6. Following reduction of peroxides, this cysteine is oxidized to a sulfenate, and GSTP participates in overcoming the accessibility barrier for GSH to reach this hydrophobic region and reactivate Prdx6. *In silico* modeling or deletion studies suggest that the site of closest contact between Prdx6 and the GSTP monomer is at the interface of their interaction at residues 115–124 for GSTP and residues 163–169 for Prdx6. Presumably, the combination of differences in molecular volume (Ala, 69Å^3; Val, 120Å^3; and Ile, 204Å^3) and hydrophobicity in the side chains impacts the affinity of GSTP for Prdx6. The affinities of GSTP1-1A (or GSTP1-1C) for Prdx6 are higher ($K_D \sim$ 51–57 nM) than those of GSTP1-1B (or GSTP1-1D) ($K_D \sim$ 101–94 nM) (71). These values determine proximity between the catalytic Cys-47 sulfenate of Prdx6 and activated GSH (thiolate) bound to the GSTP allelic variant and, consequently, the efficiency with which the sulfenate at Cys-47 is *S*-glutathionylated and reduced (*i.e.* activated) (71). The peroxidase activities of cells transiently transfected with GSTP1-1A (or GSTP1-1C) are higher than those transfected with GSTP1-1B (or GSTP1-1D), and catalytically inactive Y7F GSTP does not support activation of Prdx6 (71). These observations carry the implication that human polymorphisms of GSTP differentially mediate activation of Prdx6 and that, contingent upon their GSTP genotype, individuals will have potential differences in mounting an antioxidant response, particularly impacting protection of cell membranes against lipid peroxidation. Polymorphic variants of GSTP may also potentially impact the forward reaction in *S*-glutathionylation of other client protein clusters (72, 73). Large-scale epidemiology studies will be required to analyze the fidelity of the relationship between GSTP polymorphisms and susceptibility to ROS/RNS.

Because altered *S*-glutathionylation patterns occur in diseases characterized by redox deregulation (*e.g.* cardiovascular, lung, inflammatory, and neurodegenerative diseases and cancer), there is opportunity to use them as biomarkers. *S*-Glutathionylated hemoglobin and actin have been investigated as biomarkers in patients with Friedreich ataxia, diabetes, hyperlipidemia, and uremia associated with hemodialysis or peritoneal dialysis (74–76). In hypertension, oxidative stress-induced *S*-glutathionylation of eNOS acts as a switch providing redox regulation of cell signaling and vascular functions (52, 53). *S*-Glutathionylation of the tumor suppressor p53 has been found in human cancers, and functional inactivation of p53 by *S*-glutathionylation during ROS/RNS inhibits DNA binding in

cancer cells (77). As such, the extent of p53 *S*-glutathionylation in cancers and normal tissues exposed to chronic ROS may define a relationship between redox control of p53 and disease progression and/or therapeutic response.

Redox homeostasis and thiol regulation have been linked to hematopoiesis and the regulation of bone marrow stem cell proliferation. As a tissue, the marrow environment is separated into distinct niches characterized by adhesive molecules, cytokines, chemokines, growth factors, Ca^{2+}, and O_2 gradients that can define how hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) migrate, proliferate, and differentiate (78). Given that ROS and H_2O_2 levels impact HSC quiescence (79) and that recent data link HSC location in the distal endosteum and ROS-induced DNA damage (80), it does not seem unreasonable to propose that a redox gradient might influence HSC migration and differentiation. *S*-Glutathionylation of ER chaperone proteins regulates their function and the induction of the unfolded response (59, 81), which has roles in hematopoiesis and T and B lymphocyte differentiation (82). These types of relationships provide evidence that niche thiol status and chaperone protein *S*-glutathionylation may be involved in hematopoietic events. Interrogation of these pathways may be a productive avenue in determining how redox-altering myeloproliferative therapeutics may function (83).

In patients with ischemic heart disease and heart failure, superoxide dismutase mimetics or low doses of H_2O_2 have been used in clinical trials with bone marrow progenitor cell therapies (84, 85). Achieving effective therapeutic indices in such studies would benefit from the identification of specific biomarkers that might define the parameters that monitor either efficacy or toxicity. In this vein, accumulating evidence supports a role for serine protease inhibitor (serpins) family members in myeloproliferation and HPC mobilization (86, 87). The down-regulation of serpins A1 and A3 during cell mobilization influences the marrow microenvironment and migratory behavior of HPCs. Furthermore, radiation causes serpin A1 up-regulation in bone marrow and has been linked to inhibition of HSC and HPC mobilization (87). Serpin A1/A3 enzymatic activity is regulated by modifications of key redox-active cysteines (88). *S*-Glutathionylation of serpin A1 results in conformational changes that weaken its affinity for its target protease, thereby suppressing proteolytic activity. Serpins A1 and A3 in both mouse and human plasma are *S*-glutathionylated in a time- and dose-dependent manner when treated with a redox-modulating agent that mitigates myeloproliferation (89). In addition to regulating serpin enzymatic activity, *S*-glutathionylation of serpin family members may also influence myeloproliferation. As such, serpin *S*-glutathionylation may predict marrow response to myeloproliferative agents. Inhibition of disulfide bond formation in the redox-sensitive serpin bomapin directly affects the responsiveness of myeloid progenitor cells to their microenvironment (90). Further evaluation of protein *S*-glutathionylation in a therapeutic setting may provide the sort of biomarkers that are important in drug development and optimization of treatment strategies.

Techniques for Measuring *S*-Glutathionylation

Although methodology is improving, there are still challenges in enhancing sensitivity of detection of *S*-glutathionylation. Problems include the generally low levels of cellular *S*-glutathionylated proteins and the labile nature of protein-SSG bonds. With a putative proteome of >20,000, there is a relatively limited number of proteins (hundreds) subject to modification. Recent technical advances have facilitated enhanced sensitivity of detection, and there are informative reviews (64, 91, 92). The following methodologies are used for detection and/or quantification of *S*-glutathionylation.

Essentially two "direct" or "indirect" approaches have been used to detect the protein-SSG bond. The direct method is based on the use of radiolabeled GSH (93). Cells are preincubated with cycloheximide to block protein synthesis, incubated with [^{35}S]cysteine to radiolabel GSH, exposed to ROS, and compared with control cells (92). After removal of free radiolabeled GSH, radiolabeled *S*-glutathionylated proteins are detected and quantified by radiography. When coupled with two-dimensional gel and mass spectrometry, the site of the modification can be identified. One advantage of this approach is the *in vivo* relevance, but this method requires cycloheximide, likely perturbing cell physiology. Methods based on biotinylated GSH or membrane-permeable biotinylated reduced GSH ethyl esters have been reported (94). Tagging GSH with biotin allows detection of purified *S*-glutathionylated proteins through avidin-conjugated agarose beads followed by immunoblotting (anti-biotin antibodies) or avidin-based affinity chromatography. A prevalent method is to use commercial anti-GSH antibodies directed against the GS moiety bound to an unknown antigenic protein at an unknown site. The low sensitivity of this antibody imparts a limited detection potential, and many of the proteins identified are abundant, *e.g.* actin (95), tubulin (96), and HSP70 (93). Immunoprecipitation followed by immunoblotting with the anti-GSH antibodies can enhance detection (89).

Indirect techniques require reduction of *S*-glutathionylated proteins by Grx (6) followed by labeling the reduced protein thiol with iodoacetyl or maleimide. Derivatives of these compounds with additional mass (97), fluorescence (98), or biotin derivatives (99) in combination with LC-MS/MS, fluorescent microscopy, or immunoblotting enable labeling and monitoring of changes in redox state. Proteins are initially alkylated by *N*-ethylmaleimide to block free thiols; after reduction by Grx, these newly accessible thiols react with derivatives of iodoacetyl and maleimide groups for further application. Rapid fluorometric methods to quantify *S*-glutathionylation were developed by using GSH derivatized with 2,3-naphthalenedicarboxaldehyde (100).

Concluding Remarks and Perspectives

Cysteine is a highly malleable amino acid susceptible to many types of post-translational modifications. *S*-Glutathionylation serves both to protect and modify structure/function. The cyclical nature of the *S*-glutathionylation process allows regulation of signaling pathways to occur during variations in ROS/RNS homeostasis. So far, the number of proteins susceptible to

S-glutathionylation is not large (hundreds) compared with the proteome, but improved sensitivity of detection will likely lead to the unearthing of further candidates. There is a nascent opportunity to consider what impact protein *S*-glutathionylation may have in the cause/effect relationship of a number of human pathologies. Indeed, there are already early indications of examples in which candidate proteins may be biomarkers of exposure/response to ROS/RNS. Polymorphisms in GSTP imply that the forward reaction for *S*-glutathionylation may be subject to population differences, again raising the possibility of a link with human disease.

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