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Emerging Chemistry and Biology in Protein Glutathionylation

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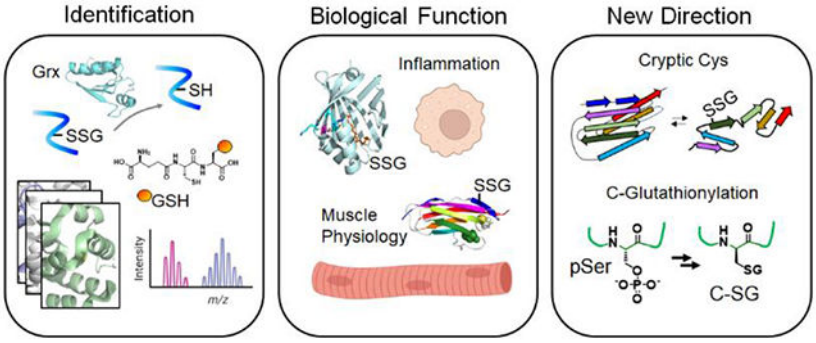
Abstract

Protein S-glutathionylation serves a regulatory role in proteins and modulates distinct biological processes implicated in health and diseases. Despite challenges in analyzing the dynamic and reversible nature of S-glutathionylation, recent chemical and biological methods have significantly advanced the field of S-glutathionylation, culminating in selective identification and detection, structural motif analysis, and functional studies of S-glutathionylation. This review will highlight emerging studies of protein glutathionylation, beginning by introducing biochemical tools that enable mass spectrometric identification and live-cell imaging of S-glutathionylation. Next, it will spotlight recent examples of S-glutathionylation regulating physiology and inflammation. Lastly, we will feature two emerging lines of glutathionylation research in cryptic cysteine glutathionylation and protein C-glutathionylation.

Graphical Abstract



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Author contributions

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Competing interests

Authors declare no competing interest.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Keywords

Cysteine; S-glutathionylation; proteomics; redox signaling; cryptic cysteines; C-glutathionylation

Introduction

Cysteine is one of the unique amino acids in proteins that retain high nucleophilicity and oxidation susceptibility in biological systems [1,2]. Due to these unique properties, cysteines play important functional roles in proteins. For example, many conserved cysteines serve as catalytic nucleophiles in enzymes (e.g., proteases), essential residues for structural folding (e.g., disulfide bond, -SS-), and metal binding [2]. Alternatively, cysteine also plays important regulatory functions in proteins via its reversible oxidations, including S-sulfenylation (SOH), S-nitrosylation (SNO), S-persulfidation (SSH), and S-glutathionylation (SSG) [3,4]. Evidence supports that individual cysteine oxoforms occur on functionally distinct protein networks and regulate different biological processes [5]. Therefore, identification and functional analyses of specific cysteine oxoforms and their complex interplay continue to be important to portray a global map of redox signaling.

Among oxoforms, protein SSG is a cysteine post-translational modification (cys-PTM) that adds bulky glutathione (GSH) to protein cysteine residues via disulfide formation, which regulates protein functions and biological processes in response to oxidants such as reactive oxygen species (ROS) [6]. As significant as ROS in physiology and pathology, the essential functions of SSG have emerged in all areas of human health, including cardiovascular regulation [6,7], inflammation and infection [8–10], apoptosis [11], and cancer [12].

At a molecular level, SSG occurs via various reaction mechanisms. SSG occurs via nucleophilic sulfur chemistry where thiolate anion (S^-) reacts with oxidized glutathione (GSSG) or reactions of GSH with electrophilic sulfur intermediates, such as sulfenic acid, S-nitrosothiol, or thiyl radical [13]. SSG formation is further balanced by the activities of several enzymes, including glutaredoxin (Grx) and glutathione transferase pi and omega (GSTP and GSTO) [14]. The complexity of SSG that forms concurrently with other cysteine oxoforms (e.g., -SS-, SOH, SNO, SSH) challenges the identification of SSG sites, which are essential to uncovering new regulatory roles of SSG. Nevertheless, recent chemical and biological methods, especially combined with chemical proteomics and mass spectrometry, have advanced the field of SSG, culminating in the identification, detection, structural motif analysis, and functional studies of SSG. This review will focus on the recent studies of SSG, highlighting 1) biochemical tools that enable the discovery and detection of SSG and 2) functional analyses of SSG in biological models. Lastly, we will feature 3) newly emerging perspectives of SSG by appraising cryptic cysteine SSG and protein C-glutathionylation (C-SG).

Emerging chemical tools to study protein S-glutathionylation

SSG can be commonly detected by using a glutathione antibody. However, recent biochemical tools enabled global and individual analyses of SSG for site identifications [15], site occupancy (i.e., SSG percentage on Cys) [16], global concurrence with other cys-PTM

[17], and cellular imaging [18]. The following section will showcase recent methods that detect and identify SSG.

Glutaredoxin (Grx)-mediated identification.

E. coli Grxs (Grx 1-3) are ubiquitous thiol-disulfide oxidoreductases with -CXXC- motifs that primarily reverse SSG (and disulfide reduction of ribonucleotide reductase) [19,20]. Mutating the resolving cysteine (the second Cys in -CXXC- motif) in *E. coli* Grx1 and Grx3 (i.e., C14S in Grx1/Grx3) confers higher specificity to deglutathionylation over disulfide reduction [21,22]. Such higher specificity of Grx1 C14S mutant (or Grx3 mutant) has been a foundation to reverse SSG selectively, which allowed selective identification of SSG among other cysteine oxidations, especially combined with the biotin-switch method (BSM) [23]. As opposed to the original BSM-based SSG detection [23], the Qian group has advanced this approach by removing a biotinylation step, thus simplifying the enrichment with the 2-thiopyridyl disulfide-based resin (Thiopropyl Sepharose), which was named as the resin-assisted capture (RAC) (Fig. 1A) [16,24,25]. In the RAC, after SSG formation, all remaining cysteines were blocked by N-ethylmaleimide (NEM). Grx1 C14S (or Grx3 C14S/C65Y in which the additional reactive cysteine, C65, was mutated to tyrosine found in other Grx isoforms [22]) was then used to reduce SSG selectively. The resulting cysteines were directly captured on the resin by forming disulfide bonds, which could reduce concerns resulting from non-specific binders of streptavidin-based enrichment. Following on-bead trypsin digestion, SSG peptides on resins were labelled by the isobaric tandem mass tag (TMT) (Fig. 1A, top). In parallel, the approach was used to capture and label all cysteine-containing peptides (SH) by TMT (Fig. 1A, bottom). TMT-based quantification of SSG versus SH samples determined levels of SSG (i.e., site occupancy) in individual cysteines (Fig. 1A) [16].

Alternatively, Grx-mediated detection of SSG has coupled with click chemistry-based polyethyleneglycol (PEG) conjugation (PEGylation) [26]. In this approach, SSG in lysates was reversed by Grx2 (mitochondrial Grx2 was used in this study, likely due to examining the mitochondrial protein). The resulting cysteines were modified by the maleimide-conjugated to trans-cyclooctene (M-TCO). The subsequent reaction with 5 kD PEG-modified methyltetrazine (Tz-PEG) enabled the detection of SSG levels in a protein via western blot analyses (Fig. 1 B). This approach was modified from a previous method that uses Cu(I)-catalyzed azide-alkyne coupling [27], which was noted to suffer from incomplete click reactions when coupling with a large size of PEG [26]. In addition, Grx-mediated detection of SSG has coupled with other detection tools, including mercury (Hg)-based enrichment or eosin-derived glutathione derivative [28,29].

The primary advantage of Grx-mediated identification is that the approach is readily applicable to biological samples from animals and patients beyond in vitro or cellular studies. For example, RAC-TMT was used to identify and quantify SSG sites upon fatiguing contraction in adult mouse skeletal muscle, finding SSG percent occupancy in 2,200 sites with an average of 4.5%, which was elevated to 5.2% upon fatiguing contraction [30]. However, potential concerns come from indirect detection of SSG in which incomplete reactions (e.g., blocking and reduction) and Grx selectivity issues during the sample

processing may compromise the outcomes. Despite such concerns, Grx-mediated SSG detection stands out as a primary tool for SSG analysis.

Chemically tagged glutathione-based identification.

Metabolic labeling of biomolecules with click chemistry reporters has been crucial for PTM analysis [31]. Our group has developed an approach to label GSH with clickable reporters (e.g., azide) (Fig. 1C) [32,33]. Glutathione synthetase (GS), a non-rate limiting enzyme in GSH biosynthesis, was engineered to produce its mutants (GS M4 or M7) that use clickable Gly derivatives (e.g., azido-Ala, allyl-Gly, allyl-Ser) in place of Gly in GSH tripeptide (γGlu-Cys-Gly) [32,34]. GS M4 enabled biosynthesis of clickable GSH (e.g., azido- or allyl-GSH) in cells, which afforded SSG analysis after click chemistry (azide-alkyne or tetrazine-alkene coupling) (Fig. 1C). The approach was also coupled with mass spectrometric proteomic analysis of SSG sites using a cleavable linker (i.e., dialkoxydiphenylsilane, DADPS), finding 1,736 SSG sites in response to hydrogen peroxide (H_2O_2) in an HL-1 cardiomyocyte cell line [35]. Recently, isotopically labelled azido-Ala (heavy or light azido-Ala with +4 or 0 Da, respectively) was developed for quantification analysis of SSG (Fig. 1C) [15] and applied to quantify levels of 1,398 SSG induced by H_2O_2 and 249 SSG in response to palmitate in a cellular model of ischemic stress, discovering SSG sites associated with muscular disorders [36]. Ma and Deng *et al.* recently used the same approach to profile the SSG proteome, including 15-hydroxyprostaglandin dehydrogenase (15-PGDH), in cluster of differentiation 38 (CD38)-mediated epithelial-mesenchymal transition [37]. The principal merit of this approach is the direct identification of SSG with glutathionyl modification on individual peptides (Fig. 1C), unlike Grx-mediated detection, thus removing the ambiguity resulting from the labile cys-PTM, which is important when considering the complexity and reversibility of cys-PTM. Also, the approach could detect SSG occurring at a basal level, as endogenous SSG formation can be detected upon adding azido-Ala without oxidative stimulus. However, the approach may not be easily adaptable to biological samples from animal or human patients, as it needs the GS M4 mutant expressed in the system. Nevertheless, GS M4-based clickable glutathione could be used in genetically engineered animals.

Mass spectrometric identification without enrichment.

Typically, the PTM-modified proteome needs enrichment before mass spectrometric analysis, which maximizes PTM detection within the resolution and sensitivity power of mass spectrometry. However, the advance in mass spectrometry has brought PTM proteomic analyses without PTM enrichment, including SSG identification [17,38,39]. One example is the top-down proteomics that uses a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer with electron-capture dissociation (ECD) [40]. The Ge group used this approach to examine sarcomeric protein PTM, including SSG, that occurs upon sarcopenia (age-dependent loss of skeletal muscle mass and function). The study found SSG of troponin I (TnI) (2-3% SSG abundance) increased in soleus skeletal muscle with older versus younger ages [39]. The approach is robust such that it enables the detection of labile PTMs and the comprehensive analyses of PTMs (i.e., different proteoforms and PTM sites and abundance), thus detecting direct glutathione conjugation (+305.03 Da) to proteoforms [39]. However, top-down proteomics has more challenges in preparing and analyzing the protein samples

(e.g., difficulty in protein-level fractionation, solubilization, and data analysis) than the peptides in bottom-up proteomics [41,42], which hampers its use. However, it is notable that the advancement in instrumentation (e.g., high-resolution Orbitrap) and the analytic technique over the years is rapidly broadening the field of top-down proteomics [43].

Alternatively, the bottom-up proteomics using the Orbitrap has advanced to direct detection of SSG and cys-PTM without enrichment [17]. Mouse pancreatic β-cell lysates were collected and analyzed after blocking nucleophilic reduced cysteines, quantifying about 231 cys-PTM sites (sulfinic acid, sulfonic acid, -SS-, SSH, and SSG; about 10 SSG) increased or decreased upon endoplasmic reticulum (ER) stress out of 9342 proteins detected in lysates [17]. The approach is significant, enabling a global view of complex cys-PTM simultaneously and estimating the relative abundance of each cys-PTM on individual cysteines. However, the approach illustrates a challenge for direct SSG detection, identifying a small number of SSG sites [17].

Imaging protein S-glutathionylation.

Despite high interest in proteome-wide identification of SSG, the approach to detect SSG in live cells has been limited. Recently, the Yao group developed nanoquencher-based probes that sense SSG formation in live cells (Fig. 1D) [18]. Mesoporous silica-based nanoparticle covalently doped with black hole quenchers (qMSN) was loaded with a fluorescent dye (rhodamine B, RhB), which emits no fluorescence due to quenchers. qMSN surface was covalently immobilized with GSH, followed by non-covalently binding to GSH antibody (Ab^{SSG}) (Fig. 1D). The large size of Ab^{SSG} acts as a gatekeeper to the pores of qMSN, blocking the release of encapsulated RhB. However, exogenous GSH or glutathionylated proteins (P-SSG) displaces the gatekeeper Ab^{SSG} from the qMSN surface, thus releasing RhB with the restoration of fluorescence (Fig. 1D). The qMSN surface was also conjugated with cell-penetrating poly(disulfide) (CPD), which mediates delivery of the qMSN-based probe into cells via a thiol-mediated uptake that enables its delivery to the cytoplasm, unlike endocytosis (Fig. 1D) [44]. Interestingly, Ab^{SSG} retains higher selectivity to P-SSG over GSH (5,000:1 selectivity), thus enabling the probe to sense a low level of P-SSG that occurs inside cells, even in the presence of a high concentration of GSH [18]. The probe is distinct from GSH probes [45,46], thus promising to contribute to SSG biology, such as high-throughput screening of small molecules in live cells that modulate levels of SSG.

Alternatively, protein A conjugated with CPD was developed for simple delivery of antibodies, including fluorescein isothiocyanate (FITC)-labelled Ab^{SSG}, namely the “Mix-and Go” approach, which enabled fluorescence imaging of SSG in live cells [47]. These approaches may progress to image SSG of a specific protein in future studies.

Emerging biological targets of protein S-glutathionylation

The identification of protein targets and sites of SSG enables understanding the functional and regulatory roles of SSG. Previous studies have found that SSG regulates diverse cellular pathways, depending on their targets. For example, individual target analysis along with proteomic analysis support that SSG regulates metabolism (e.g., glycolysis, tricarboxylic acid cycle, and the electron transport chain [48]), translation (e.g., initiation and elongation

factors [36]), signal transduction (e.g., G-protein, kinase, and phosphatase, including Ras [49], Src [50], and low molecular weight protein tyrosine phosphatase [51]), transcription factors (e.g., p53 [52]), cytoskeletal structure (e.g., actin [53,54] and vimentin [55]), inflammation (e.g., inhibitor of nuclear factor kappa-B kinase subunit β, IKKβ [56,57]), apoptosis (e.g., Fas and caspase 3 [58,59]), and calcium release (e.g., sarcoendoplasmic reticulum calcium ATPase, SERCA [60]) among others, showing a broad role of SSG in regulating biological processes. Therefore, functional analyses of SSG continue to expand our understanding of SSG in all areas of human health. This section will highlight the significance of SSG with recent examples of target proteins regulating cardiac contraction, inflammation, and coronavirus.

Titin.

Titin is an elastic myofilament protein essential for producing passive force during muscle contraction (Fig. 2A) [61]. Many studies have demonstrated that titin truncations, mutations, and PTM result in several forms of cardiomyopathy [62], supporting its critical role in heart muscle elasticity. In the structure, titin is a modular protein with immunoglobulin (Ig)-like domains and fibronectin-domains, especially with unique sequences (N2A and N2B domains) and a segment rich in proline, glutamate, valine, and lysine (PEVK domain) at the elastic I-band (Fig. 2A) [63]. The titin elasticity is governed by the unfolding and refolding of Ig-domains in addition to stretches of PEVK and unstructured inter-domain sequences [63]. Importantly, many cysteines in I-band Ig domains were found to form SSG in the unfolded state at the cryptic cysteine (Fig. 2A), which reduces dynamics of re-folding and weakens Ig-domain stability, thus decreasing titin-based passive tension or stiffness [64]. Alternatively, intramolecular disulfide (-SS-) within Ig domains hinders titin stretches, thus increasing its stiffness [65]. Recent studies support that increased sarcomere strain induces unfolded domain oxidation (UnDox) more prevalently on elastic I-band Ig-domains of titin [65]. Notably, titin SSG was found elevated, especially at I-band, in the mouse heart during ischemia, which correlated with titin phosphorylation that also reduces titin stiffness [65]. However, UnDOx, including SSG, was suggested to increase the aggregation of titin domains [65] that can cause myocardial stiffness commonly seen in heart diseases.

Fatty acid-binding protein 5 (FABP5).

FABPs are small lipid chaperons that bind hydrophobic ligands such as long-chain fatty acids and transport them to cellular compartments [66]. Guo *et al.* found that macrophage-specific Grx1 knockout alleviates inflammatory acute lung injury [67]. The subsequent Grx1-mediated SSG proteomics found FABP5 is regulated via SSG at Cys127, which can form a disulfide bond with Cys120 (see Fig. 2B). Biochemical studies support that FABP5 SSG promotes its fatty acid binding and nuclear translocation in response to ROS, activating peroxisome proliferator-activated receptor β/δ (PPAR β/δ) pro-survival target genes (Fig. 2B). Therefore, FABP5 SSG was found to suppress inflammation in macrophages and alleviate acute lung injury induced by lipopolysaccharide (LPS). It is worth noting that Cys127 is buried and positioned in the inner structure (Fig. 2B), which could disfavor SSG formation. However, Cys127 pK_a is predicted to be low (7.8 in the PropKa program [68]; in contrast, Cys120 pK_a is 12.9 using Protein Data Bank, PDB, 4LKT), supporting its potential reactivity. Notably, sulfenylation proteomics found FABP5 C127 sulfenylation elevated by

H_2O_2 [69]. In addition, chemical proteomics in *C. elegans* identified that lipid binding protein-3 (LBP-3) Cys154 (high homology to FABP5 C127) is susceptible to cysteine oxidations, modulating *C. elegans* lifespan [70].

Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC).

Inflammasomes, including NACHT, LRR, and PYD domains-containing protein 3 (NLRP3), are crucial regulators of the innate immune system in response to pathogens [71]. Inflammasome activation leads to caspase-1 activation that cleaves and activates proinflammatory cytokines (e.g., interleukin 1β and δ) (Fig. 1C), eventually inducing pyroptosis [72]. Li *et al.* found that GSTO1 knockout mice were resistant to NLRP3 inflammasome-dependent arthritis, which correlated with reduced oligomerization of ASC [73]. ASC oligomerization is necessary to bridge NLRP3 and caspase-1 to form an inflammasome complex in macrophages (Fig. 2C). ASC has only one cysteine at 171 in the caspase recruitment domain (CARD). The PropKa analysis predicts that C171 is buried but has relatively low pK_a (7.4-9.0 with PDB 6N1H) (Fig. 2C). Biochemical studies found that ASC SSG inhibits its oligomerization, resulting in the deactivation of NLRP3 inflammasome in macrophages [73]. Interestingly, mitochondrial ROS promoted GSTO1-ASC interaction, which induced GSTO1-mediated ASC deglutathionylation and NLRP3 inflammasome activation (Fig. 2C). Lastly, ASC C171A mice induced NLRP3-dependent hyper-inflammation, suggesting that ASC SSG serves as a brake to prevent NLRP3 inflammasome-induced hyper-inflammation [73].

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) main protease (M^{pro}).

Recent studies have found the potential role of GSH and SSG in coronavirus disease 19 (COVID-19) [74,75]. The causative agent, SARS-CoV-2, encodes two proteases for replication. One protease, M^{pro}, is only active as a homodimer [76]. In contrast, the monomeric active site pocket disintegrates, thus decreasing its substrate binding [76,77]. M^{pro} was found susceptible to SSG at Cys300, which is located at the dimer interface [75]. Importantly, M^{pro} Cys300 SSG inhibits its dimerization, leading to deactivation [75]. This finding highlights that M^{pro} activity is regulated by SSG, suggesting a potential therapeutic approach that targets M^{pro} Cys300.

New research direction of protein glutathionylation

The research on GSH and redox biology has brought new perspectives on SSG in recent years. This section will highlight emerging lines of SSG research, such as cryptic cysteine SSG and protein C-SG.

Conformation-dependent cryptic cysteine glutathionylation.

pK_a and residue-surface accessibility (RSA) are two determining factors for cysteine oxidations [3]. Low cysteine pK_a enables thiolate formation, increasing susceptibility to oxidation. Higher RSA increases the probability of reactions. Despite their importance, recent experimental data support low or minimal differences in average pK_a and RSA values in groups of oxidized versus non-oxidized cysteines [5,16]. Such data could arise from the limited availability of structural information. However, the discrepancy is also supported

by an emerging idea of cryptic cysteines or conformational-dependent cysteine SSG [64], defined as SSG of cysteines only accessible to oxidants upon conformational changes or potentially protein-protein interaction changes. The term cryptic cysteine was used with an example of titin [64] (Fig. 2A). In this gigantic protein, 89 out of 93 Ig-domains in the elastic I-band have cysteines buried in their β-sandwich structures [64] (e.g., two cysteines buried in the folded structure, Fig. 2A). However, these cysteines can be exposed upon unfolding of Ig-domain induced by sarcomere stretch, thus susceptible to SSG (e.g., cysteine SSG in the unfolded structure, Fig. 2A). In addition, the Held group recently reported redox regulation of cryptic cysteines that are only exposed and oxidized upon epidermal growth factor (EGF)-induced conformational changes of proteins [78], suggesting that cysteine-mediated redox signaling depends on its stimulus that alters protein structures and interactions.

Protein C-glutathionylation.

For many years, it has been observed that β-elimination of water (H_2O) and hydrogen sulfide (H_2S) from serine, threonine, and cysteine in proteins or β-elimination of phosphate from phosphoserine and phosphothreonine give rise to dehydro-amino acids, such as 2,3-dehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb) [79]. The Dha/Dhb are reactive, thus forming a thioether linkage with GSH via a Michael addition, namely C-SG [80]. The Dha/Dhb are often found in the long-lived proteins in eyes, neurons, and cardiac myocytes, thus observing C-SG in aged and cataractous lens proteins [81]. C-SG presumably protects proteins by preventing protein crosslinking and aggregation during aging and cataractogenesis [81,82]. The proteomic approach has led to C-SG identification in lens proteins [81,83].

Although C-SG is thought to occur via non-enzymatic reactions, Van Der Donk and Davis groups recently reported an example of enzyme-catalyzed C-SG formation in kinases [84]. Kinases are activated by phosphorylation at the activation loop (left box, Figure 2D). The phosphate groups at the activation loop were found to undergo β-elimination under physiological conditions, generating Dha/Dhb (middle box, Fig. 2D) [84]. Eukaryotic LanC-like proteins (LanCL) are homologous to bacterial LanC proteins that catalyze lanthionine formation between cysteine and Dha/Dhb [85]. Notably, consistent with their roles in bacteria, LanCL catalyzed C-SG formation on the activation loop of mitogen-activated protein kinase (MAPK), including mitogen-activated protein kinase kinase (MEK1) and extracellular signal-regulated kinase 1/2 (ERK1/2) (right box, Fig. 2D) [84]. LanCL-induced C-SG was specific to Dha/Dhb formed at phosphorylation sites. Therefore, the study discovers evidence of C-SG formation modulated by LanCL in the eukaryotic system.

Dha/Dhb formation at the activation loop of MEK1 was found to confer hyperactivity, while C-SG causes almost inactive MEK1 (Fig. 2D) [84]. This implied that Dha/Dhb formation in kinases causes aberrantly activated kinases, which could be rescued by C-SG formation, suggesting the protective role of C-SG. The study also identified the LanCL interactome, implying that LanCL quenches deleterious effects of Dha/Dhb in many damaged proteins (namely eliminylome, proteins bearing β-elimination) [84]. Pre-mature death of LanCL knockout mice further demonstrates the protective role of C-SG [84].

Therefore, discovering C-SG mediated by LanCLs in kinases unfolds a new research area in irreversible glutathionylation.

Conclusion

SSG is a major form of reversible cysteine oxidation responsive to the cellular redox environment modulated by oxidases, redox enzymes, and metabolic states [36,86]. The continuing research has significantly advanced the field of SSG in recent years. Proteome-wide analyses found about 4-5% basal levels of SSG (and ca. 10% basal cysteine oxidation) in proteins [16], suggesting their responsive capacity upon stimulus. Chemical proteomics has discovered many SSG sites (>2,000 in database) in the proteome [15,30,35,87]. Functional analyses of individual proteins unveiled SSG in regulating protein structure and functions. Although not included here, many examples of animal models unraveled the biological roles of enzymes regulating SSG [88–92]. Lastly, examples of conformation-dependent cryptic cysteines and C-SG provide a new venue to appraise SSG biology. Future studies will continue developing new approaches and uncovering SSG biology, culminating in discovering therapeutic opportunities.

Acknowledgments

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Highlights

- Cysteine glutathionylation serves distinct regulatory roles in proteins
- Various biochemical tools have been developed for the identification, detection, and imaging of glutathionylation.
- Proteomic strategies for site specific identification and site occupancy analyses of glutathionylation are available.
- Functional analysis of glutathionylation spans all areas of human health, including cardiac regulation, inflammation, and infection.
- Evidence for cryptic cysteine glutathionylation and irreversible C-glutathionylation is emerging.

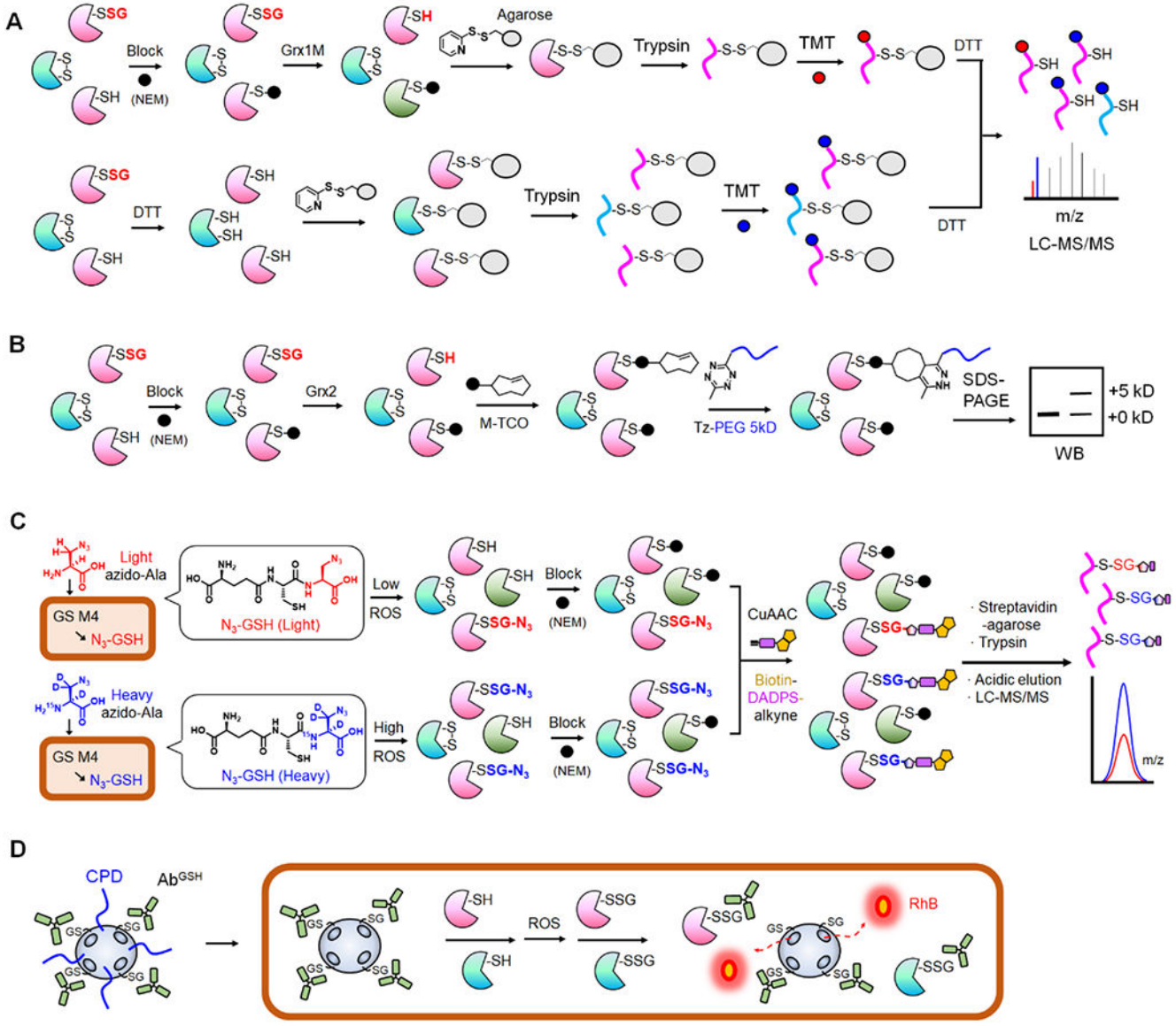

Figure 1. Biochemical methods for identification and detection of SSG.

A. Grx-mediated detection of SSG or RAC-TMT. SSG is selectively reduced by Grx1 mutant (Grx1M) after blocking Cys with N-ethylmaleimide (NEM, black circle). Disulfide-based agarose captures reduced Cys in proteins (top). In parallel, all Cys-containing peptides from the same sample are captured on agarose after reducing with dithiothreitol (DTT, bottom). After trypsin digestion and TMT labeling, the bound peptides are eluted and combined for LC-MS/MS analysis to determine SSG levels. B. Grx-mediated SSG analysis with click chemistry. After selective reduction of SSG by Grx, the revealed Cys is reacted with maleimide-conjugated trans-cyclooctene (M-TCO). The subsequent reaction with 5-kD PEG-conjugated tetrazine (Tz-PEG) enables analysis of SSG levels via western blot. C. Glutathione synthetase mutant (GS M4)-based clickable glutathione. GS M4 uses light or heavy azido-Ala to synthesize isotopically labelled azido-GSH (N_3-GSH) in cells. Isotopically labelled glutathionylated proteins (P-SSG) are combined, modified with

biotin-DADPS-alkyne by click chemistry, captured on streptavidin-agarose, and digested by trypsin. The acidic cleavage of the DADPS linker enables elution of SSG-modified peptides for relative quantitation of SSG. D. Nanoparticle to image SSG in live cells. Mesoporous nanoparticle doped with black hole quenchers (qMSN) encapsulates and quenches rhodamine B (RhB). qMSN is modified with GSH on the surface, which binds to the GSH antibody (Ab^{GSH}) that blocks the release of RhB. The cell-penetrating disulfides (CPD) of qMSN enable its cell entry where SSG proteins (P-SSG) cause dissociation of Ab^{GSH} and releases of RhB, restoring fluorescence.

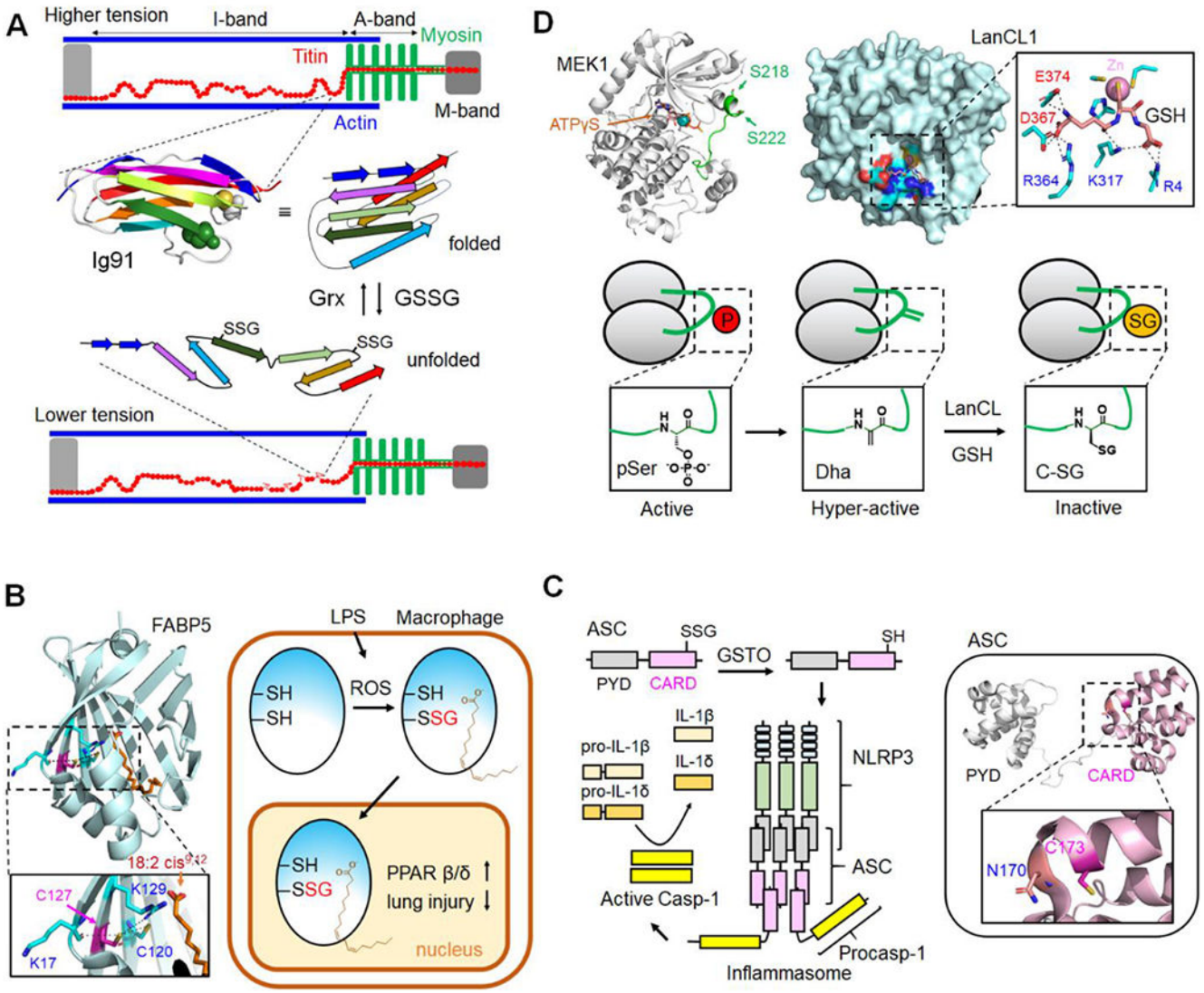

Figure 2. Examples of SSG in biology.

A. Titin SSG in muscle contraction. Titin has Ig-domains, including Ig91 (two cysteines in a space-filling model, PDB: 1TIT), at the elastic I-band that can unfold during sarcomere stretches. Ig-domain unfolding exposes cryptic cysteines susceptible to SSG. SSG in Ig-domain retards refolding and promotes its relaxed state, which reduces the titin-based passive tension. B. FABP5 SSG in macrophage. FABP5 C127 (pink in β-strand, PDB: 4LKT) is susceptible to SSG in response to LPS-induced ROS, promoting fatty acid binding and translocation to the nucleus. FABP5 in the nucleus activates PPAR β/δ genes for pro-survival, suppressing macrophage inflammation. C. ASC SSG in inflammasome formation. LPS-ATP or mitochondrial ROS promotes GSTO interactions with ASC, causing its deglutathionylation. ASC deglutathionylation enables its oligomerization, which bridges and forms an inflammasome with NLRP3 and pro-caspases. Inflammasome complex activates caspase-1, which processes and activates cytokines for pyroptosis. ASC has C173 in the human CARD domain (PDB: 2KN6, equivalent to mouse C171). D. LanCL catalyzed C-glutathionylation (C-SG) in kinases. The phosphate sites (S218 and S222) in the

activation loop (green) of MEK1 (PDB: 3EQD) and other kinases are susceptible to forming Dha/Dhb at physiological conditions, which cause hyperactivation of kinases. Dha/Dhb at the activation loop reacts with GSH to form C-SG catalyzed by LanCL, which inactivates the hyperactivity of kinases, thus preventing kinase dysregulation. The LanCL active site has a binding site for GSH (PDB: 3E73).