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Mining the Thiol Proteome for Sulfenic Acid Modifications Reveals New Targets for Oxidation in Cells

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ABSTRACT Oxidation of cysteine to sulfenic acid has emerged as a biologically relevant post-translational modification with particular importance in redox-mediated signal transduction; however, the identity of modified proteins remains largely unknown. We recently reported DAz-1, a cell-permeable chemical probe capable of detecting sulfenic acid modified proteins directly in living cells. Here we describe DAz-2, an analogue of DAz-1 that exhibits significantly improved potency *in vitro* and in cells. Application of this new probe for global analysis of the sulfenome in a tumor cell line identifies most known sulfenic acid modified proteins: 14 in total, plus more than 175 new candidates, with further testing confirming oxidation in several candidates. The newly identified proteins have roles in signal transduction, DNA repair, metabolism, protein synthesis, redox homeostasis, nuclear transport, vesicle trafficking, and ER quality control. Cross-comparison of these results with those from disulfide, *S*-glutathionylation, and *S*-nitrosylation proteomes reveals moderate overlap, suggesting fundamental differences in the chemical and biological basis for target specificity. The combination of selective chemical enrichment and live-cell compatibility makes DAz-2 a powerful new tool with the potential to reveal new regulatory mechanisms in signaling pathways and identify new therapeutic targets.

Historically, reactive oxygen species (ROS) have been considered solely as toxic byproducts of metabolism. However, a growing body of work suggests that oxidants can also act as a second messenger in signal transduction pathways that regulate normal cellular processes such as cell growth, differentiation, and migration (*1–3*). Early studies by Sundaresan *et al.* showed that hydrogen peroxide (H_2O_2) generation was required for platelet-derived growth factor signal transduction (*4*). Later, Lee *et al.* reported that peptide growth factors stimulate H_2O_2 production in epithelial cells and that ROS transiently inactivate protein tyrosine phosphatase 1B (PTP1B), implicating oxidants in post-translational modification (PTM) of proteins in a manner analogous to regulatory *O*-phosphorylation (*5*). The subsequent finding of superoxide-generating homologues of the phagocytic NADPH oxidase (Phox) by Suh *et al.* revealed the enzymatic source of ligand-stimulated ROS production (*6*). From these seminal discoveries, the field of redox signaling in mammalian cells emerged.

The signaling roles and sources of ROS are well established; however, the specific intracellular protein targets of oxidation remain largely unknown. Emerging evidence suggests that redox-sensitive cysteine residues in proteins may function as oxidant sensors (*7–9*). Oxidation of sulfur alters its chemical reactivity and metal-binding properties, which can serve as a molecular switch to control protein structure and function (*10, 11*). In addition, thiols exhibit a wide range of reactivity with H_2O_2 ($1-10^7$ M^{-1} s^{-1}), which may account in part for target selectivity (*1*). The initial oxidation product of cysteine and H_2O_2 is sulfenic acid. This cysteine oxoform resides at a hub and can be reduced back to the thiol

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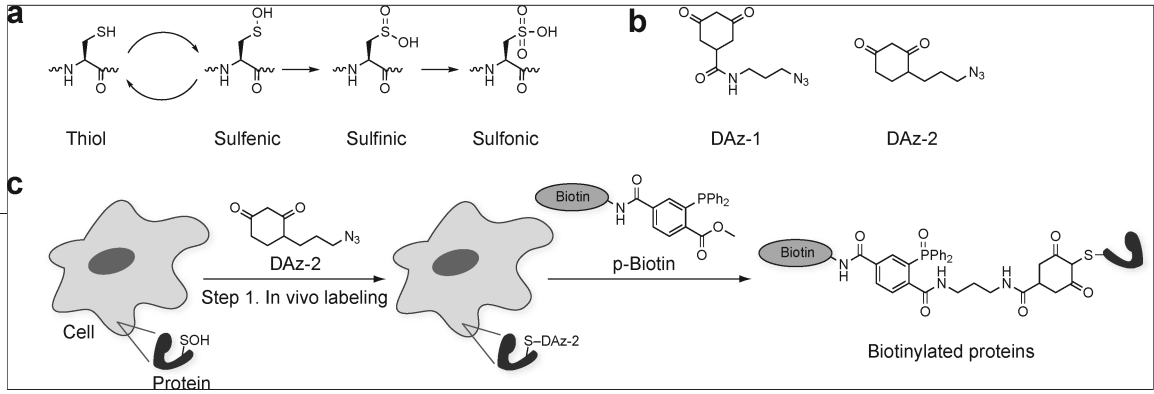


Figure 1. Trapping and tagging proteins that undergo sulfenic acid modification, directly in living cells. a) Oxidation states of protein cysteines that are implicated in biological function. b) Azido-probes for detecting and identifying sulfenic acid modified proteins, depicted in their keto form. c) Two-step strategy for detecting cellular sulfenic acid modifications.

or converted to sulfinic and sulfonic acid, which are interesting oxidation states in their own right (*12*) (Figure 1, panel a). Stable sulfenic acids have been observed in a growing number of proteins (*13–18*). Furthermore, essential functions for sulfenic acid have been demonstrated for redox signaling in yeast and T-cell activation, providing strong support for the growing roles of this modification in biology (*9, 19*).

The chemical reactivity of sulfenic acids has been well studied in the past several decades (*20–22*). The sulfur atom in sulfenic acid has both electrophilic and nucleophilic characteristics. This is underscored by the facile reaction that occurs between sulfenic acids to form a thiosulfinate. Sulfenic acids also undergo nucleophilic conjugate addition reactions. The chemoselective reaction between 5,5-dimethyl-1,3-cyclohexadione (dimedone) and protein sulfenic acids was first reported by Benitez and Allison in 1974 (*17*). Since then, this reaction has been exploited to detect protein sulfenic acids using mass spectrometry (MS) (*23, 24*) and, more recently, through direct conjugation to fluorophores or biotin (*25–27*). Nonetheless, these previous approaches have limited utility in live-cell proteomic labeling for a variety of reasons, which include (i) membrane impermeability, (ii) the presence of endogenously biotinylated proteins, and/or (iii) lack of a chemical handle for affinity enrichment.

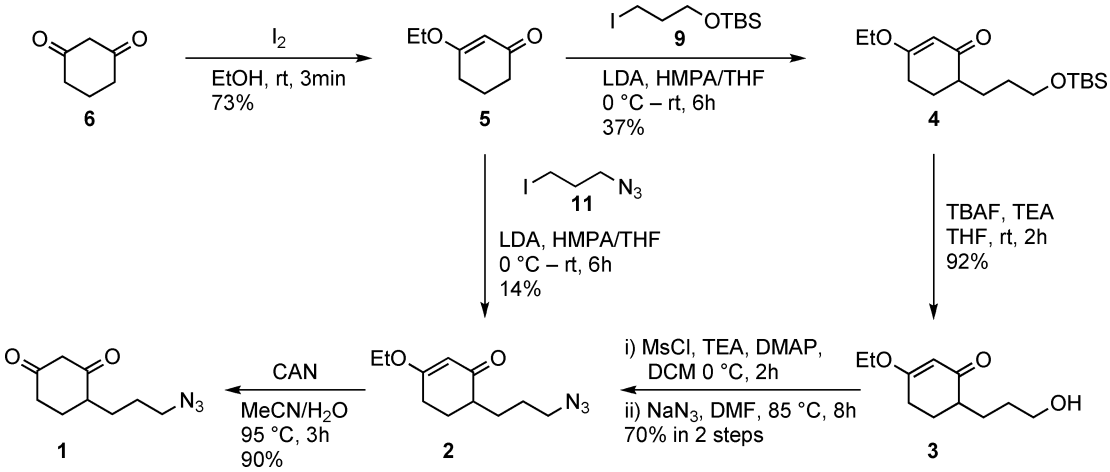
In particular, live-cell investigation is vital to expand our understanding of the role that sulfenic acids and other oxidative cysteine modifications play in biological systems. In large part this is due to redox compartmentalization in cells, consisting of highly oxidizing organelles such as the endoplasmic reticulum and peroxisomes amidst the cytoplasm and nucleus, which represent very reducing compartments (*28, 29*). Cell lysis destroys this delicate redox balance, producing hyperoxidized proteins and making it difficult to decipher modifications that occur under biologically relevant conditions (*28, 29*). To address these issues, we recently reported on the development of DAz-1 (Figure 1, panel b), a bifunctional chemical probe that is cell-permeable and chemoselective for sulfenic acids (*30, 31*). In addition, DAz-1 incorporates the azide chemical reporter group that permits selective coupling with bioorthogonal phosphine or alkyne-derivatized reagents for subsequent detection of oxidized proteins (*32, 33*).

In the present study, we report a new probe for sulfenic acid detection, 4-(3-azidopropyl)cyclohexane-1,3-dione (**1**) or DAz-2 (Figure 1, panel b), an analogue of DAz-1 with improved potency *in vitro* and in living cells. Application of this new probe for global analysis of the "sulfenome" in a tumor cell line, using the strategy outlined in Figure 1, panel c, identifies most known sulfenic acid modified proteins, 14 in total, plus more than 175 new candidates, with further testing confirming oxidative modifications in vesicle-targeting GTPase and ER glycoprotein chaperone candidate proteins. The newly identified proteins have roles in signal transduction, DNA repair, metabolism, protein synthesis, redox homeostasis, nuclear transport, vesicle trafficking, and ER quality control, demonstrating broad cellular manifestations of this oxidative modification. Cross-comparison of these results with those from disulfide, *S*-glutathionylation, and *S*-nitrosylation proteomes reveals moderate overlap, suggesting fundamental differences in the chemical and biological basis for target specificity.

RESULTS

We previously designed and synthesized DAz-1 (Figure 1, panel b), the first chemical probe capable of trapping and tagging sulfenic acid modified proteins directly in living cells (*30, 31*). DAz-1 comprises two key components: (i) the 1,3-cyclohexadione scaffold, which is chemically selective for sulfenic acids (*17, 25–27, 30, 31, 34*), and (ii) an azide chemical handle that allows selective conjugation to phosphine or alkyne-derivatized reagents for subsequent analysis of labeled proteins (*32, 33*). DAz-1 selectively tags sulfenic acid modified proteins *in vitro* and in living cells; however, this probe is less well suited for global proteomic studies because its reactivity is decreased relative to dimedone (not

SCHEME 1. Synthesis of DAz-2, a new cell-permeable probe for detecting sulfenic acid-modified proteins in cells



shown). In addition, the polar amide group attached to the C5 position of DAz-1 could reduce cell permeability or accessibility to partially buried sulfenic acids. To extend the utility of our two-step labeling approach to detect cellular sulfenic acids, we sought an analogue of DAz-1 with increased potency. Such a probe would allow us to more comprehensively profile and define the cellular sulfenome. To address these issues, we envisioned an analogue of DAz-1, 4-(3-azidopropyl)cyclohexane-1,3-dione or DAz-2 (**1**), with a propyl azide linker installed directly at the C4 position of the 1,3-cyclohexadione scaffold (Figure 1, panel b).

Synthesis of DAz-2. We explored the synthesis of DAz-2 *via* two routes (Scheme 1). The synthesis of DAz-2 *via* route A began with protection of the reactive diketone of 1,3-cyclohexadione (**6**). The protected 3-ethoxycyclohex-2-enone (**5**) was generated with iodine in ethanol in 73% yield. A lithium diisopropylamide (LDA) solution was then used to activate **5** for alkylation with 3-iodo-*tert*-butyldimethylsilyloxypropane (**9**) to give **4** in 37% yield. Compound **4** was then deprotected with tetrabutylammonium fluoride (TBAF) to give the primary alcohol **3** in 92% yield (*27*). The azide chemical handle was installed *via* mesylation of 6-(3-hydroxypropyl)-3-ethoxycyclohex-2-enone (**3**) and subsequent nucleophilic displacement of the mesyl alcohol by sodium azide in 70% yield, over two steps. The resulting 6-(3-azidopropyl)-3-ethoxycyclohex-2-enone (**2**) was deprotected using ceric(IV) ammonium nitrate (CAN) in water/acetonitrile in 90% yield. The overall yield of DAz-2 over 6 steps was 16%. In an attempt to reduce the number of synthetic steps we synthesized DAz-2 by the alternative route B (Scheme 1). A lithium diisopropylamide (LDA) solution was used to activate **5** for alkylation with 3-iodo-propylazide (**11**) (*35*) to give **2** in 14% yield. Compound **2** was then deprotected to give the final product DAz-2 in 9% overall yield over 3 steps.

Comparative Analysis of Sulfenic Acid Labeling by DAz-1 and DAz-2 in Recombinant Protein and Whole Cell Lysate. The chemical selectivity of DAz-1 and C4-functionalized 1,3-cyclohexadione analogues for sulfenic acid has been well established (*17, 25–27, 30, 31, 34*). To test the hypothesis that DAz-2 would be more effective at sulfenic acid detection than DAz-1, we compared their reactivity using glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme with an active site cysteine, Cys149, that is readily oxidized (*17, 36*). For these experiments untreated or H_2O_2-treated GAPDH was reacted with DAz-1 or DAz-2. In subsequent steps, excess probe was removed; azide-tagged proteins were conjugated to phosphine-biotin (p-Biotin) and analyzed by HRP-streptavidin Western blot (Figure 2, panel a). In reactions without H_2O_2, faint labeling of GAPDH by DAz-1 and DAz-2 was observed and is consistent with the high reactivity of Cys149, which oxidizes in the presence of trace metal ions under aerobic

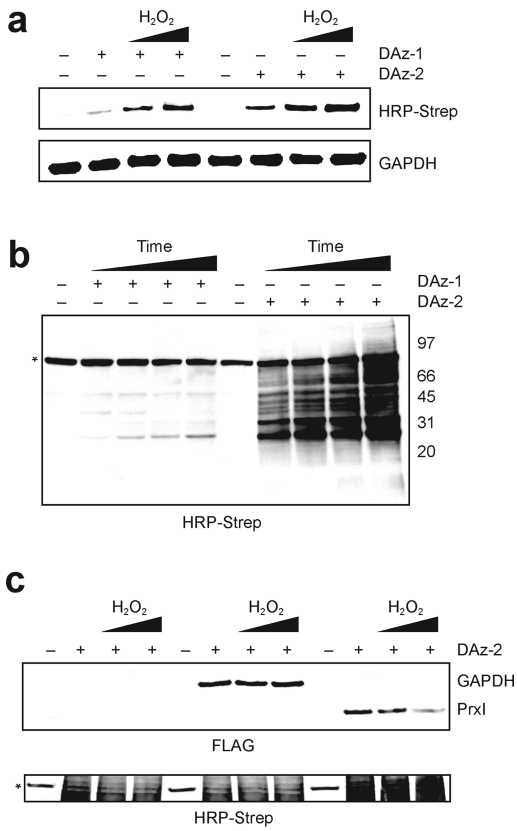


Figure 2. DAz-2 exhibits increased sensitivity for sulfenic acids *in vitro*. a) Comparison of sulfenic acid detection by DAz-1 and DAz-2 in the glycolytic enzyme GAPDH. Protein (10 μM) was reacted with or without H_2O_2 (10 or 50 μM) for 20 min at RT and then with DAz-1 (1 mM), DAz-2 (1 mM), or DMSO (5% v/v) for 15 min at RT. Reactions were quenched with DTT (5 mM), and excess reagent was removed by gel filtration. After Staudinger ligation (see Methods), proteins were resolved by 4–12% SDS–PAGE, transferred to PVDF membrane and detected by HRP-streptavidin Western blot (see Methods). b) Comparison of sulfenic acid detection by DAz-1 and DAz-2 in HeLa whole cell lysates. Protein lysate (50 μg) was labeled with DAz-1 (0.5 mM), DAz-2 (0.5 mM), or DMSO (5% v/v) for 0.5, 1, 1.5, and 2 h at 37 °C. After labeling, excess reagent was removed by gel filtration, and samples were analyzed as described above. c) DAz-2 detects sulfenic acid modifications in lysate from cells expressing FLAG and HA epitope-tagged GAPDH and PrxI. Protein lysate (200 μg) was treated with or without H_2O_2 (50 or 100 μM) for 15 min and then with DAz-2 (1 mM) or DMSO (2% v/v) for 1 h at 37 °C. After Staudinger ligation, biotinylated proteins were captured on NeutrAvidin beads, processed, and analyzed by Western blot using anti-FLAG antibody and HRP-streptavidin (see Methods). The asterisk marks proteins that are endogenously biotinylated.

conditions (*17*, *36*). Control reactions carried out in the absence of DAz-1 or DAz-2 demonstrated no detectable protein labeling. Addition of H_2O_2 and concomitant oxidation of GAPDH resulted in a marked increase in protein labeling, as expected. Notably, protein labeling of GAPDH with DAz-2 is more intense relative to DAz-1. Next, we compared the reactivity of DAz-1 and DAz-2 in HeLa whole cell lysates using the procedure outlined for GAPDH. Sulfenic acid detection by the azido-sulfenic acid probes was time- (Figure 2, panel b) and dose-dependent (not shown); protein-labeling patterns were also similar. Analogous to the results obtained with GAPDH, DAz-2 proved more effective at sulfenic acid detection in lysates, as indicated by the prominent increase in signal relative to DAz-1.

Having demonstrated that DAz-2 was more effective at detecting sulfenic acids *in vitro*, we next tested our strategy to enrich labeled proteins from lysate prepared from cells expressing N-terminally FLAG and HA epitope-tagged GAPDH and Peroxiredoxin I (PrxI), an antioxidant protein with a redox-active cysteine that is oxidized to sulfenic acid by its peroxide substrate (*37*) (Supplementary Figure S1). Untreated or H_2O_2-treated lysates were probed with DAz-2 and azide-tagged proteins conjugated to p-Biotin. Biotinylated proteins were then captured on immobilized NeutrAvidin, a modified avidin derivative (*38*). After extensive washing, bound proteins were eluted and analyzed by Western blot using a monoclonal anti-FLAG antibody and HRP-streptavidin (Figure 2, panel c). The extent of GAPDH enrichment did not parallel the increase in exogenously added H_2O_2 and confirms that complete oxidation of GAPDH occurred during cell lysis (*28*). On the other hand, PrxI enrichment decreased concomitantly with elevated H_2O_2, suggesting that in the absence of a functional reducing system, the active site cysteine formed an intramolecular disulfide or was overoxidized to sulfinic acid, precluding reaction with DAz-2 (*37*). These data confirm DAz-2-dependent capture of oxidized GAPDH and PrxI, as compared to cells expressing an empty vector control, and underscore the importance of probing labile cysteine modifications directly in cells.

Specific Labeling of Sulfenic Acid-Modified Proteins in Cells Using DAz-2. DAz-2 was then evaluated for detecting sulfenic acid modifications in living cells. In these studies, the extent and selectivity of covalent modification of sulfenic acid modified proteins was examined by performing the Staudinger ligation in lysates

from cells treated with azido-probe. Protein labeling by DAz-2 was time- (Figure 3, panel a) and dose-dependent (Supplementary Figure S2, panel a). Cells probed using DAz-1 or DAz-2 exhibited similar protein-labeling patterns with higher signal intensity obtained with cells treated with DAz-2, as anticipated on the basis of *in vitro* observations. Upon addition of a moderate concentration of H_2O_2 to cells (200 μM, Supplementary Figure S2, panel b), we observed an increase in protein labeling by DAz-2. Mock-treated cells displayed only low levels of background labeling from HRP-streptavidin binding to endogenous biotinylated proteins (Figure 3 and Supplementary Figure S2). These data establish that DAz-2 is membrane-permeable and can respond to changes in protein sulfenic acid formation within cells. Control experiments performed with the cell-permeable oxidant-sensitive probe 2′,7′-dichlorofluorescein diacetate (DCF-DA) showed that DAz-2 treatment does not increase intracellular ROS levels (Supplementarty Figure S3, panel a). In addition, no difference in the ratio of reduced to oxidized glutathione (GSH/GSSG) was found between untreated and DAz-2-treated cells (Supplementary Figure S3, panel b). Moreover, no increase in the inactive forms of PrxI-IV, detected using anti-Prx-SO3 antibodies that recognize both sulfinic and sulfonic forms of overoxidized Prx (*39–41*), was observed over the course of DAz-2 treatment (Figure 3, panel a). Lastly, trypan blue exclusion studies confirm that cell viability was not significantly affected by incubation with 5 mM DAz-2 for 2 h at 37 °C (Supplementary Figure S3, panel c). Taken together, the data indicate that DAz-2 treatment does not trigger endogenous oxidative stress or cell death.

Next, we investigated whether DAz-2 could monitor changes in the cellular oxidation state of the thiol peroxidase PrxI. Cells expressing epitope-tagged PrxI or a C173A variant (predicted to stabilize the sulfenic acid intermediate at the peroxidatic cysteine (*37*)) were untreated or exposed to H_2O_2, probed with DAz-1 or DAz-2, and subjected to the enrichment procedure outlined above. Immobilized proteins were then analyzed by Western blot using a monoclonal anti-HA antibody and HRP-streptavidin (Figure 3, panel b and Supplementary Figure S1). In DAz-2-treated cells, the amount of wild-type PrxI isolated on solid phase paralleled the increase in H_2O_2 concentration, as expected (Figure 3, panel b). This trend was reversed in PrxI C173A, which exhibited a more intense labeling relative to the wild-type enzyme

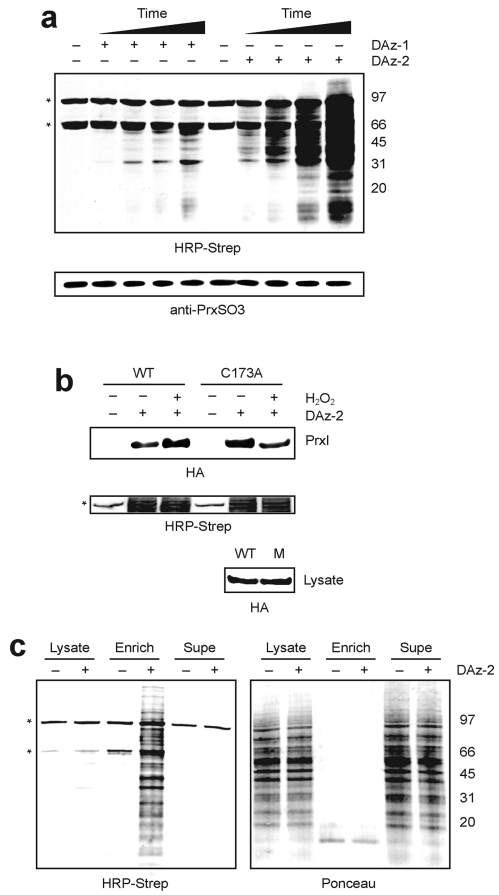


Figure 3. DAz-2 exhibits increased sensitivity for sulfenic acids in living cells. a) Comparison of cellular sulfenic acid detection by DAz-1 and DAz-2. HeLa cells were treated with DAz-1 (5 mM), DAz-2 (5 mM), or DMSO (2% v/v) for 0.5, 1, 1.5, and 2 h at 37 °C, harvested in lysis buffer (see Methods), and processed as described for Figure 2, panel a above. In order to examine endogenous oxidative stress, the Western blot was reprobed using an antibody that recognizes the sulfinic and sulfonic acid forms of Prxs I—IV (see Methods). b) DAz-2 detects sulfenic acid modification of wild-type and C173A PrxI directly in cells. HeLa cells expressing FLAG and HA epitope-tagged wild-type or C173A PrxI were treated with or without H_2O_2 (400 μM) for 15 min at RT and then with DAz-2 (5 mM) or DMSO (1% v/v) for 2 h at 37 °C. Cells were harvested in lysis buffer and processed as described in Figure 2, panel c above, except that anti- FLAG was replaced by anti-HA antibody. c) Representative example of enrichment of proteins labeled by DAz-2 in cells. HeLa cells were treated with DAz-2 (5 mM) or DMSO (1% v/v), processed as described in Figure 2, panel c above, and detected by HRP-streptavidin Western blot. The asterisk marks proteins that are endogenously biotinylated.

TABLE 1. Abbreviated list of identified proteins with sulfenic acid modifications in HeLa cells[a]

Protein	Location[b]	Function	Previous identification[c]	Reference
		DNA repair		
Ku80	N	Double-stranded DNA damage repair, helicase		
MCM6	N	DNA replication initiation		
80 kDa MCM3-associated protein	C, N	DNA replication, acetyltransferase		
		Metabolism		
Amino acid transporter E16	C, PM	Cationic amino acid transporter		
Catechol-*O*-methyltransferase	C, PM	Transfers methyl group from SAM to catecholamines		
C1-tetrahydrofolate synthase	C, M	One-carbon metabolism		
Glyceraldehyde-3-phosphate dehydrogenase	C	Glycolysis	X	*17, 48, 52, 54, 64*
Glucosidase α	ER, G	*N*-Glycan biosynthesis		
Iron−sulfur protein assembly 1 homologue	C, N	Iron−sulfur cluster assembly, transcription regulator	X	*54*
Neutral amino acid transporter B	PM	Neutral amino acid transporter		
		Nuclear transport		
Importin 9	C, N	Regulates nuclear transport and interacts with PP2A		
Karyopherin β 1	C, N	Interacts with protein nuclear localization signals		
Ran	C, N	Regulates nuclear transport and DNA synthesis	X	*54, 110*
		Protein homeostasis		
Calnexin	ER	Ca^{2+} binding, ER chaperone		
Calreticulin	C, ER, N	Ca^{2+} binding, ER chaperone, regulator of transcription	X	*45*
ER-Golgi intermediate compartment protein 1	ER, G	Upregulates protein turnover		
Hs7p75 (TRAP1)	M	Mitochondrial chaperone		
Hsp90-α	C	Stress-inducible chaperone	X	*63*
Ubiquitin-activating enzyme E1	C, N	Catalyzes the first step in ubiquitin conjugation	X	*111*
		Protein synthesis		
Gln-tRNA synthetase	C	Protein synthesis and biogenesis of rRNA	X	*49*
hnRNP U protein	N	mRNA splicing		
Met-tRNA synthetase	C, NU, PM	Protein synthesis and biogenesis of rRNA		
Ribosomal protein S3	C, N	Protein synthesis and regulator of DNA repair		
Ribosomal protein S6	C, N, NU	Protein synthesis and TOR signaling		
Thr-tRNA synthetase	C	Protein synthesis and biogenesis of rRNA		
U2 small nuclear RNA auxiliary factor 1	N	mRNA splicing		

TABLE 1. Abbreviated list of identified proteins with sulfenic acid modifications in HeLa cells[a], continued

Protein	Location[b]	Function	Previous identification[c]	Reference
		Redox homeostasis		
Glutathione transferase Mu 3	C	Detoxification of electrophiles by conjugation to glutathione	X	*48*
Peroxiredoxin I	C, N	Peroxide catabolism and regulator of cell proliferation	X	*37, 45, 48*
		Signaling		
Aspartyl β-hydroxylase	ER	Hydroxylates aspartic acid residues in EGF-like domains		
Cardiotrophin-like cytokine factor 1	S	Cytokine-mediated signaling		
IQGAP1	C, PM	Regulates small GTPase activity		
G protein, β polypeptide 2	C, PM	Regulatory heterotrimeric G protein subunit		
PI-3-kinase, catalytic subunit	C, M	Phosphorylates 3′-hydroxyl of inositol		
Protein GPR107	PM	G-protein coupled receptor		
Protein phosphatase PP1, catalytic subunit	C	Dephosphorylates serine and threonine residues		
SH2 domain-containing adapter protein F	C	Phosphotyrosine recognition		
		Vesicle transport		
Rab1a	C, ER, G	Regulates ER to Golgi vesicle transport		
Rab10	C, G, PM	Regulates vesicle traffic through early endosomes		
Rab33b	C, G	Modulates autophagosome formation		

[a]Peptides from proteins with probability values of $P \leq 0.05$ were considered statistically significant. [b]C, cytoplasm; ER, endoplasmic reticulum; G, Golgi; M, mitochondria; N, nucleus; NU, nucleolus; PM, plasma membrane; S, secreted. [c]Previous identification as redox-active whether oxidized or *S*-thiolated (with the corresponding references).

in the absence of exogenous oxidant, consistent with an increase in basal oxidation level in the variant protein (Figure 3, panel b). DAz-2 incorporation in PrxI C173A decreased in cells exposed to H_2O_2 (Figure 3, panel b). These findings suggest that in the absence of a resolving cysteine the peroxidatic cysteine overoxidizes to the sulfinic and sulfonic acid forms, which are not detected with dimedone-based probes. Collectively, these results establish that DAz-2 is capable of detecting changes in the oxidation state of PrxI in cells.

Global Detection and Proteomic Analysis of Sulfenic Acid-Modified Proteins in Cells. Having demonstrated the improved potency of DAz-2 labeling *in vitro* and in cells, we proceeded to use the reagent for global detection and proteomic analysis of sulfenic acid modified proteins. HeLa human tumor cells were chosen for these studies owing to their routine use in culture and high intrinsic level of oxidative stress, which is thought to drive cell proliferation and immortalization (*42*). To identify sulfenic acid modified proteins, cells were treated with DAz-2, and azide-tagged proteins were subjected to Staudinger ligation using p-Biotin and subsequently purified by NeutrAvidin beads. Labeled proteins were separated by SDS−PAGE, digested in-gel with trypsin, and extracted, and the resulting peptides were subjected to LC−MS/MS analysis for protein identification. Figure 3, panel c shows a representative example of affinity-purified proteins obtained from DAz-2 *versus* mock-treated cells. A protein sequence database search using the MS/MS data led to the identification of 193 proteins (Table 1 and Supplementary Table S1) with a diverse range of biological functions (Figure 4, panel a),

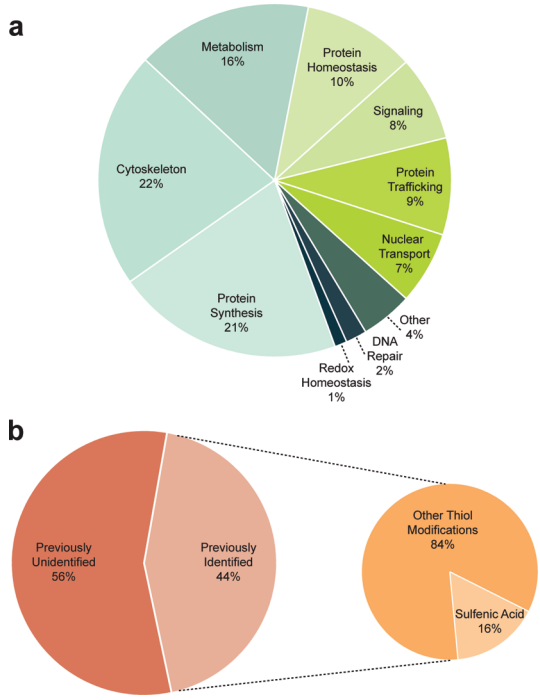


Figure 4. Analysis of candidates identified in our proteomic experiments with HeLa cells. a) All candidates grouped on the basis of protein function. Because some proteins are involved in multiple processes, some generalizations were necessary. b) Percentage of candidates previously identified as having redox-active cysteines that undergo oxidation to sulfenic acid or other oxidative cysteine modifications including disulfide, *S*-glutathionylation, and *S*-nitrosylation.

cellular distribution, and abundance. Of these candidate proteins 44% (85 of 193) are known to harbor redox-active cysteine residues subject to disulfide, glutathionyl, nitrosyl, or sulfenic acid modification (or some combination thereof) (Figure 4, panel b) (*25, 43–54*). Among the 44% known redox-active proteins, 16% (14 of 85) have been confirmed to undergo sulfenic acid modification, including PrxI and GAPDH (Figure 4, panel b) (*17, 37*). The identification of proteins previously known to have redox-active cysteines confirms the ability of DAz-2 for efficient isolation and identification of sulfenic acid modifications. To the best of our knowledge, the remaining 56% (108 of 193) of candidates identified in our study have not been previously established as having redox-active cysteines. Finally, 93% (179 of 193) were previously unknown to undergo sulfenic acid modification (Figure 4, panel b).

***In Vitro* Validation of Selected Candidate Proteins.** To further confirm that our methodology targets sulfenic acid modified proteins labeled by DAz-2 in cells, we examined the ability of the azido-probe to detect oxidation in two candidates, Rab1a and calreticulin. Rab1a is a small GTPase that regulates protein transport between the Golgi and endoplasmic reticulum (ER) (*55*). The primary sequence of Rab1a contains four cysteine residues (Cys26, Cys126, Cys204, and Cys205). Of these, two are constitutively geranylgeranylated in cells (Cys204 and Cys205) (*56*), which leaves the remaining two residues, Cys26 and Cys126, as potential targets for oxidation. Experiments were performed with a variant form of Rab1a lacking the -XXCC prenylation motif, which was produced in bacteria and purified to homogeneity (hereafter referred to as wild-type Rab1a). When oxidized protein was reacted with DAz-2, an intense signal was observed by HRP-streptavidin Western blot analysis (WT, Figure 5, panel a), which establishes that Rab1a undergoes sulfenic acid modification. To identify the site or sites of modification we explored a panel of Rab1a variants—C26A, C126A, and the C26A, C126A double mutant (DM)—in further detail (Figure 5, panel a). Oxidized Rab1a proteins were treated with DAz-2, and probe incorporation was assessed, as described above (Figure 5, panel a). Protein labeling was observed for C26A and C126A Rab1a. However, the C126A variant showed a substantial decrease in labeling relative to that in wild-type and C26A Rab1a. Removal of Cys26 and Cys126 abolished DAz-2 incorporation, and control reactions with protein minus DAz-2 gave no background signal, as expected. Together, these data show that Rab1a undergoes sulfenic acid modification at Cys126 and, to a lesser extent, at Cys26. Next, we investigated sulfenic acid formation in the candidate protein calreticulin, a multifunctional calcium-binding chaperone in the ER that functions in quality control and folding of newly synthesized (glyco)proteins (*57*). The primary sequence of calreticulin contains three conserved cysteines, two of which form a disulfide bridge. On the basis of homology modeling with a related chaperone, calnexin (*58*), the remaining cysteine resides in the globular N-terminal domain, which interacts with newly synthesized protein substrates. Experiments were performed with commercially available calreticulin owing to the difficulty of production in bacteria. Labeling of calreticulin by DAz-2 in the absence of exogenously added peroxide was observed (Figure 5, panel b), indicating

that the redox-sensitive cysteine was already modified to sulfenic acid. Exposure to high concentrations of H_2O_2 abolished labeling of calreticulin (presumably due to overoxidation to sulfinic and sulfonic acid), and pretreatment with dimedone, prior to the addition of azido-probe, produced a significant reduction in signal. Taken together, these results establish that Rab1a and calreticulin undergo sulfenic acid modifications that can be detected by DAz-2 *in vitro* and further confirm our findings in living cells.

DISCUSSION

Redox-active cysteine residues are highly conserved in protein sequences and are present in all three domains of life: eukaryotes, prokaryotes, and archaea. A recent and particularly elegant computational study predicts redox-active cysteines in 10,412 unique sequences among all completed genomes, which can be further organized into 40 protein families and superfamilies with significant functional diversity (*59*). A small amount of these proteins are known to undergo sulfenic acid modification *in vitro*, which raises a fundamental question: can all redox-active cysteines in proteins be modified to sulfenic acid? Most certainly, the answer to this question depends on contextual factors such as environment as well as the nature of the oxidant and its concentration. An important follow-up question is: of the cysteines that undergo sulfenic acid modification, which of these modifications have functional relevance in cells? Pivotal roles for sulfenic acid modification in redox-regulated signal transduction have been proposed by many (*2, 7, 8*); however, progress in this area and the investigation of other cysteine oxoforms, has been limited due in large part to the lack of chemical tools to trap and tag labile, oxidative modifications directly in cells. Clearly, then, addressing the questions posed above requires us to devise new methods for sulfenic acid detection. Recent technological advances in detection of cellular ROS underscores the promise of selective and live-cell-compatible reagents to broaden our understanding of redox-signaling pathways (*60*).

Inspired by earlier chemical approaches (*17, 27, 61, 62*), the development of DAz-1 represented an important step forward in cellular sulfenic acid detection (*30, 31*). However, the modest reactivity of DAz-1 made the reagent less suited for global proteomic studies. For this reason we designed and synthesized DAz-2, a cell-permeable probe that detects sulfenic acids more effectively *in vitro* (Figure 2) and in living cells (Figure 3). Differences in reactivity between DAz-1 and DAz-2 could be due to several factors including polarity, nucleophilicity, and stability of the probes. The log *P* values of DAz-1 and DAz-2 are 0.02 and 0.5, respectively (see Supplemental Methods), and thus the increased hydrophobicity of DAz-2 could enhance its membrane permeability. It is also possible that the decreased polarity and size of DAz-2 could facilitate access to partially buried protein sulfenic acids. The nucleophilic carbons in DAz-1 and DAz-2 have almost identical ^{13}C NMR chemical shifts (103.5 and 103.4 ppm, respectively), indicating similar electronic environments. Lastly, the amide bond of DAz-1 could be cleaved by cellular amidases and thus preclude Staudinger ligation. Additional experiments,

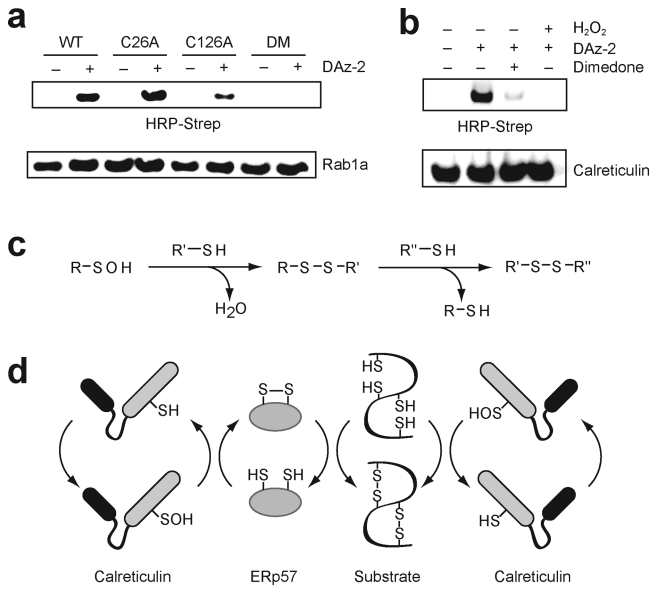


Figure 5. *In vitro* validation of DAz-2 labeling in selected candidates, Rab1a and calreticulin, and hypothetical role for oxidizied calreticulin in protein folding. a) Rab1a undergoes sulfenic acid modification at C26 and C126. Oxidized wild-type and cysteine variants of Rab1a (10 μM) were treated with DAz-2 (1 mM) or DMSO (10% v/v) for 15 min at RT and processed as described for Figure 2, panel a above. b) DAz-2 detects sulfenic acid modification of calreticulin. Protein (1 μM) was treated with dimedone (10 mM), H_2O_2 (1 mM), or buffer for 0.5 h at RT and then with DAz-2 (1 mM) or DMSO (10% v/v) for 15 min at RT. Reactions were processed as described for Figure 2, panel a above. c) Sulfenic acids can condense with a nearby cysteine thiolate to generate a disulfide, which is then subject to thiol—disulfide exchange. d) Oxidized calreticulin may drive protein folding by promoting disulfide formation in ERp57 (left). Alternatively, oxidized calreticulin could directly promote disulfide formation in target proteins (right).

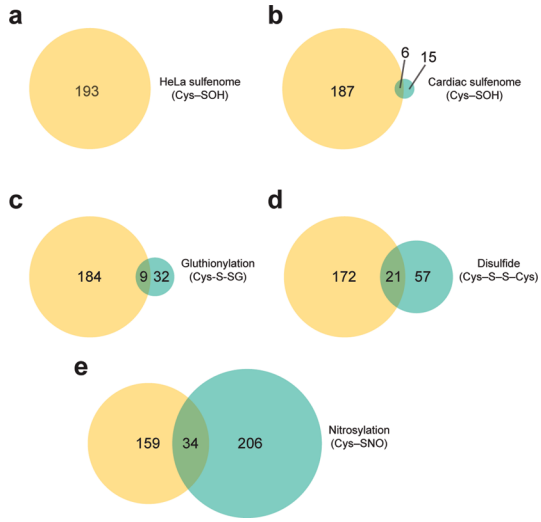


Figure 6. Venn diagrams of proteins sensitive to sulfenic acid formation in HeLa cells (a) compared to prior sulfenic acid (*25*) (b), *S*-glutathionylation (*46*) (c), disulfide (*45*) (d), and *S*-nitrosylation (*48*) (e) proteomic studies. The circle area is proportional to the number of proteins identified in each study.

including a detailed analysis of the reaction mechanism, will be required to identify the precise factors that underlie the observed differences in reactivity.

Using DAz-2 and the strategy outlined in Figure 1, we conducted global proteomic analysis of the sulfenome in the HeLa tumor cell line. This study resulted in the identification of 193 proteins (Table 1; Supplementary Table S1; Figure 6, panel a) including the majority of known sulfenic acid modified proteins, 14 in total, with more than 175 new candidates (Table 1 and Supplmentary Table S1), with further verification in Rab1a and calreticulin candidate proteins (Figure 5, panels a and b). The newly identified proteins have roles in signal transduction, DNA repair, metabolism, protein synthesis, redox homeostasis, nuclear transport, vesicle trafficking, and ER quality control (Figure 4, panel a), which suggests broad cellular manifestations of this oxidative modification. The implications of these findings will be discussed in turn.

Comparative Analysis of Related Modifications and Proteomic Studies. The Venn diagrams in Figure 6 provide a comparative analysis of overlap among candidate proteins identified in the current study with those reported in previous redox thiol proteomic studies (*25, 44, 46, 48*). [For the sake of comparison, mammalian studies reporting the largest compendium of proteins in each category of modification are included in Figure 6. Many other excellent thiol redox proteomic studies have been reported (*25, 43–48, 50, 52–54, 63–67*) and are included, as appropriate, in Table 1 and Supporting Information.] Using a biotinylated derivative of dimedone, Charles *et al.* identified a collection of 21 cytoskeletal and metabolic proteins in peroxide-treated rat ventricular myocytes that undergo sulfenic acid modification (*25*). Of these proteins, six out of the 19 whose expression is not confined to cardiac tissue were also identified by our study, providing independent verification and validation for these candidates (Figure 6, panel b). Addition of a biotin tag to dimedone precludes membrane permeability (*31, 61, 62*) and may account for the relatively modest number of proteins identified in this prior study (*25*).

Many redox-active cysteines undergo more than one kind of oxidative modification (*63*). We therefore compared candidates identified in this study with *S*-glutathionyl (*46*) (Figure 6, panel c), disulfide (*44*) (Figure 6, panel d), and *S*-nitrosyl (*48*) (Figure 6, panel e) proteomes in eukaryotic cells. *S*-Glutathionylation is another physiological, reversible modification of protein cysteine residues defined by the addition of the tripeptide glutathione (GSH) to form a mixed disulfide (Cys-S-SG) (*68*). *S*-Glutathione adducts are hypothesized to protect cysteine residues from overoxidation and can be formed *via* many routes including attack of a redox-active cysteine on oxidized glutathione (GSSG) or condensation of a protein sulfenic acid with GSH (*69*). Assessment in human T lymphocytes using ^{35}S-labeled glutathione revealed 41 proteins with *S*-glutathione modifications (*46*), and 9 of these proteins overlapped with candidates identified in the current study (Figure 6, panel c). Disulfide bonds are formed from the oxidation of two thiol groups (Cys-S-S-Cys) and play an important role in folding and stability of proteins that reside in oxidizing environments (*63*). In addition, reversible disulfide bond formation serves a regulatory function in many proteins, including transcription factors (*9, 70*). Proteomic analysis of intermolecular disulfide formation in rat ventricular myocytes *via* two-dimensional PAGE analysis identified 78 candidates (*44*), 21 of which are present in the current study (Figure 6, panel d). A complementary study reported by Cumming *et al.* looking at both intra- and intermolecular disulfide formation in HT22 cells identified 47 candidate proteins (*45*), 19

of which were also found in the current study. Finally, *S*-nitrosylated proteins (Cys-SNO) form when cysteine reacts with nitric oxide in the presence of an electron acceptor and in transnitrosylation reactions (*71, 72*). Similar to H_2O_2, nitric oxide has been identified as a second messenger in signal transduction cascades and modulates many biological processes (*73, 74*). A recent investigation of *S*-nitrosylation in human spermatozoa using the ascorbate-mediated biotin switch technique identified 240 candidate proteins (*48*), 34 of which were also identified in the present study (Figure 6, panel e).

As illustrated by the comparisons above, a subset of the proteins identified in our study undergo an array of oxidative cysteine modifications. For example, the glycolytic enzyme GAPDH has been found in virtually all redox proteomic studies reported to date (Table 1), which indicates a high level of promiscuity in oxidative cysteine modification for this protein. However, it is also important to emphasize that not all cysteine residues in proteins are readily oxidized and not all low pK_a thiols undergo multiple modifications (*75*). Indeed, the majority of proteins in the current study have not been identified in *S*-glutathionyl, disulfide, or *S*-nitrosyl proteomic studies (Table 1; Supplementary Table S1; Figure 4, panel b). Although much remains to be understood about the features of a cysteine residue that favor oxidation by H_2O_2, our data suggest that some proteins preferentially undergo sulfenic acid modification in cells. To this end, protein structure and cellular environment are likely to play a critical role in defining target specificity.

Protein Candidates for Sulfenic Acid Modification. The protein targets of sulfenic acid modification identified in this study are found in many cellular locations and have diverse roles in signal transduction, DNA repair, metabolism, protein synthesis, redox homeostasis, nuclear transport, vesicle trafficking, and ER quality control (Table 1; Supplementary Table S1; Figure 4, panel a).

Signal Transduction. Although considerable evidence implicates ROS as mediators of cellular signaling, the molecular mechanisms by which ROS alter these pathways are not well-known. In receptor tyrosine kinase (RTK)-mediated signal transduction pathways, reversible inactivation of protein tyrosine phosphatases (PTPs) (*7*) and changes in the activity of kinases such as Src (*76, 77*) have been accredited to tightly regulated growth factor and cytokine-stimulated ROS generation. In the present study, a number of proteins with pivotal roles in signal transduction have been identified as new candidates for redox-regulation (Table 1 and Supplementary Table S1) such as phosphoinositide-3-kinase (PI3K) and IQGAP1, both of which are intimately connected to RTK signaling (*78*). IQGAP1 is proposed to interact with the NADPH oxidase Nox2 to enhance ROS production in endothelial cells, and under conditions of oxidative stress, IQGAP1 localizes to the cell membrane where it regulates Src activity (*79*). We have also identified serine/threonine-protein phosphatase (PP1), a regulatory protein downstream of PI3K in insulin signaling (*80*). While oxidation of the active site cysteine in the family of PTPs has been well established (*5, 81*), redox-regulation of serine/threonine phosphatases is not well understood (*82*).

DNA Repair and Replication. Recent studies estimate that $\sim 2 \times 10^4$ ROS-related DNA damaging events occur in a typical human cell every day (*83*). Given this statistic, it is perhaps not surprising that DNA repair proteins Ku80 and MCM6 were identified in this study (Table 1). *In vitro* studies have shown that Ku-DNA binding activity is redox-sensitive and requires reduced thiols (*84, 85*). However, no disulfide bonds have been observed in the crystal structure of free Ku or the Ku-DNA complex (*86*). It light of our findings, it is possible that oxidation of key cysteine(s) in Ku80 to sulfenic acid, and possibly higher states, may regulate DNA binding activity. To our knowledge, oxidative modification of minichromosome maintenance (MCM) proteins has not been previously reported, and additional studies will be required to elucidate the potential role of cysteine oxidation in these proteins.

Metabolism. Glycolytic enzymes such as aldolase, enolase, pyruvate kinase, and GAPDH were identified in the present study (Table 1 and Supplementary Table S1). GAPDH, pyruvate kinase, and other glycolytic enzymes have also been identified in a proteomic study of low pK_a cysteines (*66*). Oxidative inactivation of GAPDH is proposed to function as a cellular switch to redirect carbohydrate flux and promote generation of the reduced electron carrier nicotinamide adenine dinucleotide phosphate (NADPH), an important cofactor for cellular antioxidant enzymes (*87*).

Protein Synthesis. Studies in yeast show that oxidative stress causes the dissociation of actively translating ribosomes and that this response is mediated, in part, by the mitogen-activated protein kinase, Rck2 (*88*).

On the other hand, *in vitro* translation studies show that direct exposure of translational machinery to oxidants such as H_2O_2 increases the rate of protein synthesis (*89*). In the present study, we have identified a subset of ribosomal proteins and aminoacyl tRNA synthetases as candidates for sulfenic acid modification (Table 1 and Supplementary Table S1). Ribosomal proteins of the 40S and 60S subunits and heterogeneous nuclear ribonucleoproteins (hnRNPs) are predicted to harbor redox-active cysteines in their RNA recognition motifs (*51*). However, oxidation of these residues has not been verified experimentally. Another recent study of reversibly oxidized proteins in yeast uncovered redox-active cysteines in elongations factors and components of the 60S ribosomal complex (*66*). These data provide independent confirmation for the candidates in the current study and suggest that cysteine oxidation may play an important regulatory role in protein synthesis.

Redox Homeostasis. Two proteins with known roles in redox homeostasis were identified in our study: the antioxidant enzyme PrxI and glutathione *S*-transferase (GST), a detoxifying protein that catalyzes the conjugation of reduced glutathione to electrophilic agents in the cell (Table 1) (*90*). GST is known to form intermolecular disulfides in response to oxidative stress inactivating its normal maintenance functions (*91*). Disulfide formation in GST might be accelerated by condensation of the sulfenic acid, or this modification may serve a different function altogether. By contrast, the ubiquitous thioredoxin (Trx) and glutaredoxin (Grx) antioxidant enzymes, which also harbor reactive cysteines, were not identified in this study. It is possible that intramolecular disulfide formation between the cysteines in their CXXC redox active site motifs outcompetes reaction of DAz-2 with the sulfenic acid form of these proteins.

Nuclear Transport. Numerous *in vitro* and cellular studies show that oxidative stress disrupts nuclear transport (*92*–*95*), and Ran, a regulatory GTPase, has two redox-active cysteine residues (C85 and C120) that undergo nitrosylation *in vitro* (*93*). In support of these prior observations we have identified Ran as a protein that undergoes sulfenic acid modification in cells (Table 1). Additional candidate proteins with roles in nuclear import and export were identified (Table 1 and Supplementary Table S1) including transportin 1, a member of the importin-β family, previously found in a disulfide proteomic study (*45*).

Vesicle Trafficking. In this study we also identified a subset of Rab GTPase proteins, which function as key regulators of vesicle transport in eukaryotes (Table 1 and Supplementary Table S1) (*96*). The Rab family of proteins (~60 encoded in the human genome) is part of the Ras superfamily of small GTPases. Previous work shows Ras can be *S*-nitrosylated and *S*-glutathionylated within a conserved NKCD motif, leading to an increase in Ras activity and to downstream signaling (*97*, *98*). Another sequence motif, GXXXXGK(S/T)C, is subject to oxidation in the related Rho GTPase (*99*). In our study, we identified a total of 10 Rab GTPases. Among these, five possessed the NKCD motif, and nine contained the GXXXXGK(S/T)C motif. Additional experiments performed with recombinant Rab1a, which harbors both sequence motifs above, confirmed sulfenic acid modification of both cysteines, Cys26 and Cys126, in the GXXXXGK(S/T)C and NKCD motifs, respectively (Figure 5, panel a). While both single variants exhibited DAz-2 labeling, C126A exhibited the greatest decrease in modification (Figure 5, panel a). It is possible that cysteine oxidation in Rab GTPases might regulate membrane association by modulating their catalytic cycle and/or protein−protein interactions *via* modification-associated conformational changes.

Chaperone-Mediated Protein Folding. Chaperone proteins belonging to the major 90, 70, and 60 kDa families were also identified as targets of DAz-2 in cells (Table 1 and Supplementary Table S1). No prior evidence of sulfenic acid modifications of these proteins exists, however, and a subset of these proteins have been reported to undergo *S*-nitrosylation (*48*). Overexpression of Hsp75 is associated with a decrease in ROS production and helps maintain mitochondrial membrane potential during glucose deprivation of astrocytes (*100*). Hsp70 and Hsp90 possess a redox-sensitive cysteine in close proximity to their ATP binding pockets (*101*), and under conditions of oxidative stress, peptide binding to Hsp70 and complex stability are enhanced (*102*).

The molecular chaperones calnexin and calreticulin were also identified in our study. Additional experiments performed with calreticulin show that this chaperone contains a highly redox-active cysteine that undergoes sulfenic acid modification (Figure 5, panel b). Although the precise site of oxidation was not explored in the current study, the most likely candidate is the free cysteine C146 (*103*), located within the substrate-

interacting N-terminal domain. Calreticulin interacts with the protein-disulfide isomerase homologue ERp57 to promote oxidative folding of nascent glycoproteins in the ER lumen (*104–106*). To date, however, the role of calreticulin in glycoprotein folding has been relegated solely to binding target proteins (*57, 107*). The presence of a redox-active cysteine in calreticulin suggests that this chaperone may play additional roles in protein folding and in promoting the formation of protein disulfides (Figure 5, panels c and d). Sulfenic acids can condense with a nearby cysteine thiolate to generate a disulfide, which is then subject to thiol-disulfide exchange (Figure 5, panel c). By extension, oxidized calreticulin might drive protein folding by promoting disulfide formation in ERp57, (Figure 5, panel d, left). In support of this hypothesis, the yeast homologue of calreticulin, Cne1p, enhances the activity of a yeast oxireductase Mpd1p *in vitro* (*108*). Alternatively, oxidized calreticulin could directly promote disulfide formation in target proteins (Figure 5, panel d, right). To this end, small-angle X-ray studies show that calreticulin can dimerize to form an additional binding site capable of interacting with peptides (*109*). We also note that calreticulin is also found in the nucleus, where it interacts with the DNA-binding domain of various nuclear receptors and modulates gene transcription (*57*). Thus, it is possible that oxidation of redox-active cysteine residues in calreticulin might also modulate this particular cellular function.

Conclusion. In summary, we have described DAz-2, a new chemical tool for selective detection of protein sulfenic acids in cells. Using this reagent, we have conducted the first global proteomic study of proteins that harbor sulfenic acid modifications in cells. Candidates identified from this study are distributed in locations throughout the cell and function in a diverse array of biological processes. These findings suggest that sulfenic acid modifications may play broader biological roles than previously thought and highlight the need for future research in this emerging field. The ability to detect protein sulfenic acid modifications in living cells provides a powerful strategy for mapping redox-regulated signaling networks in normal physiological functions and disease.

METHODS

Synthesis of DAz-2. Experimental procedures are provided in Supplementary Methods.

Cloning, Expression, and Purification of GAPDH, PrxI, Rab1a. Experimental procedures are provided in Supplementary Methods.

Staudinger Ligation. Staudinger ligation was carried out by incubation of purified protein or cell lysate (1 mg mL^{-1}) with p-Biotin (200 μM) and DTT (5 mM) for 2 h at 37 °C with agitation. Reactions in lysate were quenched by the addition of cold acetone (1 mL), incubated at −80 °C for 20 min and collected by centrifugation at 4 °C for 20 min (17,000*g*). The protein pellet was washed once with cold acetone (200 μL) and then resuspended in 0.2% sodium dodecyl sulfate in phosphate-buffered saline (SDS-PBS).

Western Blot. Biotinylated proteins were separated by SDS−PAGE using Criterion XT 4−12% Bis-Tris gels (BioRad) or NuPAGE Novex 4−12% Bis-Tris Midi gels (Invitrogen) and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). After transfer, the PVDF membrane was blocked with 3% BSA or 5% milk in phosphate-buffered saline Tween-20 (PBST) overnight at 4 °C or 1 h at RT. The membrane was washed with PBST (2 × 10 min) and then incubated with 1:10,000 to 1:100,000 HRP-streptavidin. PVDF membrane was washed with PBST (2 × 5 min, 1 × 10 min) and then developed with ECL Plus chemiluminescence (GE Healthcare). To verify equal protein loading, GAPDH was probed with 1:1,000 anti-GAPDH (Santa-Cruz) and 1:35,000 rabbit anti-mouse-HRP (Invitrogen). To assess endogenous oxidative stress, hyperoxidized Prxs were probed with 1:2,000 anti-Prx-SO3 antibody (Abcam), which recognizes the sulfinic and sulfonic acid forms of PrxI-IV, and 1:100,000 goat anti-rabbit-HRP (Invitrogen). Membranes were routinely stained with Ponceau S to assess quality of protein transfer and loading.

Cell Culture. HeLa cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C and cultured in DMEM media supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin-L-glutamine (PSG), and 1% MEM nonessential amino acids (MEM-NEAA).

Preparation of HeLa Cell Lysates. HeLa cells (~90% confluent in 10 cm dishes) were washed with sterile PBS (×3), and the plate was incubated on ice for 5 min. Cells were then gently scraped with a rubber policeman and transferred to an eppendorf tube. Cold lysis buffer (100−150 μL) (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40) containing 2× PI was added, and the tube was incubated on ice for 20 min with frequent mixing. The mixture was then centrifuged at 4 °C for 20 min (17,000*g*). The supernatant was collected and used immediately or flash-frozen and stored at −80 °C. DAz-1-, DAz-2-, or DMSO-treated cells were washed with PBS (×3) prior to addition of lysis buffer.

Enrichment of Biotinylated Proteins. NeutrAvidin-coated beads (Pierce) were incubated with biotinylated proteins for 2 h at 4 °C and washed [×3 PBS, ×1 RIPA (50 mM Tris HCl, pH 7.4, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40), ×1 with 0.2% SDS-PBS and ×1 with PBS]. Proteins were eluted from NeutrAvidin in 40 μL of elution buffer EB (50 mM Tris HCl, pH 7.4, 1% SDS, 1 mM biotin) with heating to 95 °C for 5 min.

Isolation of DAz-2-Labeled Proteins for MS Analysis. HeLa cells (8 × 10 cm dishes) were grown to 90−95% confluence. Replicate samples of approximately 5 × 10^6 cells were resuspended in 0.5% FBS DMEM (4 mL) containing DAz-2 (5 mM) or DMSO (1% v/v). Cells were incubated for 2 h at 37 °C with frequent mixing and harvested at 1,000*g* for 5 min. In addition, cells were routinely evaluated for viability before and after treatment

(Supplementary Figure S2). After washing with PBS (×3), cells were incubated for an additional 1 h in complete DMEM. Lysates were generated, precleared with NeutrAvidin beads (20 μL) for 25 min at 4 °C, subjected to Staudinger ligation (1 mg mL^{-1}), and quenched with cold acetone (1 mL). Protein pellets were harvested and washed with cold acetone (200 μL) before complete resuspension in PBS containing 0.2% SDS. NeutrAvidin beads (60 μL) were incubated with lysate (500 μL) for 2 h at 4 °C, collected by centrifugation at 2,500*g* for 1 min, and washed (×3 PBS, ×1 RIPA, ×1 PBS), and proteins were eluted in EB by heating at 95 °C for 10 min. Eluents were collected, concentrated by vacuum evaporation (SpeedVac), and resuspended in water (38 μL) with SDS−PAGE loading dye. Samples were boiled at 95 °C for 5 min, resolved by SDS−PAGE, and visualized by staining with Colloidal Blue (Invitrogen). Proteins were digested in-gel with trypsin and extracted, and the resulting peptides were subjected to LC−MS/MS analysis. MS/MS data were analyzed using the MASCOT sequence database; peptides with probability values of $P \leq 0.05$ were considered statistically significant and included in Tables 1 and Supplementary Table S1.

Detection of Sulfenic Acid Modifications in Rab1a and Calreticulin. Sulfenic acid modification of recombinant wild-type, C26A, C126A, and C26A C126A Rab1a GTPase was verified by incubating protein (10 μM) with DAz-2 (1 mM) or DMSO (10% v/v) in PBS for 15 min at RT. Sulfenic acid modification of calreticulin was verified by incubating protein (Stressgen, 1 μM) with DAz-2 (1 mM) or DMSO (10% v/v) in 50 mM Tris, 50 mM NaCl, 5 mM $CaCl_2$, pH 7.1. In some reactions, calreticulin was pretreated with dimedone (10 mM) for 0.5 h at RT and then DAz-2 (1 mM). Alternatively, calreticulin was exposed to H_2O_2 (1 mM) for 0.5 h at RT and then DAz-2 (1 mM). Proteins were resolved by SDS−PAGE and analyzed by HRP-streptavidin Western blot.

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Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

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