Bioorthogonal Chemical Reporters for Analyzing Protein Sulfenylation in Cells

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ABSTRACT

Protein sulfenylation (RSOH), the redox-based modification of cysteine thiol side chains by hydrogen peroxide (H_2O_2), is an important mechanism in signal transduction. Likewise, dysregulated protein sulfenylation contributes to a range of human pathologies, including cancer. Efforts to elucidate the diverse roles of protein sulfenylation in physiology and disease have been hampered by the lack of techniques to probe these modifications in native environments. To address this problem, selective chemical reporters have been developed for the detection and identification of sulfenylated proteins directly in cells. In the approach described here, a cyclic β-diketone warhead is functionalized with an azide or alkyne chemical handle. An orthogonally functionalized biotin or fluorescent reporter is then appended to the probe post-homogenization via click chemistry for downstream analysis. These bi-functional probes are exquisitely selective for protein sulfenyl modifications, non-toxic, and do not perturb intracellular redox balance. These reagents have been utilized to investigate sulfenylation in vitro and to identify intracellular protein targets of H_2O_2 during cell signaling. These methods provide a facile way to detect protein sulfenic acids and to study the biological role of cysteine oxidation with regard to physiological and pathological events. *Curr. Protoc. Chem. Biol.* 4:101-122 © 2012 by John Wiley & Sons, Inc.

Keywords: thiol modification • protein sulfenylation • redox signaling • click chemistry • in-gel fluorescence • western blotting

INTRODUCTION

Although historically viewed as purely harmful, recent studies demonstrate that hydrogen peroxide (H_2O_2) functions as an important physiological regulator of intracellular signaling pathways, including cell growth and proliferation (Rhee, 2006; Dickinson and Chang, 2011). In this context, H_2O_2 is produced by a family of membrane-bound NADPH-dependent oxidases whose expression is tightly regulated, compartmentalized, and tissue-specific (Lambeth, 2004). The downstream effects of H_2O_2 are mediated by covalent modification of cysteine residues found within redox-sensitive target proteins (Reddie and Carroll, 2008; Paulsen and Carroll, 2010). Oxidation of these specific and reactive cysteine residues in turn can lead to modulation of protein activity, much like *O*-phosphorylation (Rhee et al., 2000).

Sulfenic acid (RSOH), also known as sulfenylation, is the direct product of cysteine modification by H_2O_2. The reactivity of a cysteine thiol toward H_2O_2 is profoundly influenced by the protein environment. Important factors include accessibility and proximity to side chains that may interact with the thiol, peroxide, or the oxidized product. Most reactive are cysteine residues with low pK_a values that exist as the nucleophilic thiolate anion at physiological pH. However, other factors are undoubtedly involved, as the documented reactivity of protein thiolate anions with H_2O_2 ranges across seven orders of magnitude (1 to 10^7 $M^{-1}sec^{-1}$) (Winterbourn, 2008). Some of these factors are being revealed, but

consensus motifs have not yet emerged and the understanding of the molecular basis of sensitivity toward H_2O_2 for a given cysteine residue remains largely ill-defined.

Once formed, sulfenyl modifications can be stabilized by the microenvironment or, in some cases, react with nearby protein or exogenous thiols to form disulfides (Fig. 1A). Indirect evidence for the formation and stabilization of sulfenic acid in proteins was first reported for the glycolytic enzyme GAPDH in the 1970s (Benitez and Allison, 1974), but was not fully appreciated until detailed biochemical and structural analysis of NADH peroxidase in the late 1990s (Yeh et al., 1996; Crane et al., 1997). These landmark studies led to the discovery that sulfenyl groups function as catalytic redox centers (Wood et al., 2003), alter enzyme chemistry (Salmeen et al., 2003), and act as reversible sensors of H_2O_2 levels in microbial transcription factors (Zheng et al., 1998). Under conditions of oxidative stress, sulfenyl groups can also oxidize further to sulfinic ($-SO_2H$) or sulfonic acid ($-SO_3H$). On the other hand, mounting evidence also points to the existence of specialized enzymes that mediate the direct reduction of the sulfenyl modification back to the thiol state (Depuydt et al., 2009).

Historically, methods to detect reactive cysteines and H_2O_2-mediated oxidation of protein thiols required homogenization of cells, which disrupts the native environment (Leonard and Carroll, 2011). Under these crude conditions, cellular redox balance and protein structure are compromised, leading to artifactual oxidation during sample processing. The severity of this issue cannot be disregarded and greatly increases the challenges associated with identifying sites of modification in low-abundance proteins, as well as in interpreting the biological significance of the data.

To overcome this problem, small-molecule probes that enable sulfenylated proteins to be trapped and tagged directly in cells have been developed (Reddie et al., 2008; Leonard et al., 2009; Paulsen et al., 2011). In this approach, a cyclic β-diketone warhead (**2**; Fig. 1C) that is chemically selective for sulfenic acids known as dimedone (Benitez and Allison, 1974) (**1**; Fig. 1B) is functionalized with an azide (**3** to **5**; Fig. 1C) or alkyne (**6** to **8**; Fig. 1C) reporter group. An orthogonally functionalized biotin or fluorescent tag is then appended post-homogenization for detection via the Staudinger ligation (Saxon and Bertozzi, 2000) or Huisgen [3+2] cycloaddition (i.e., Click chemistry) (Rostovtsev et al., 2002). In addition, two complementary isotope-coded probe pairs to quantify changes in protein sulfenylation (**9**, **10;** Fig. 1C) have been developed (Seo and Carroll, 2011; Truong et al., 2011). Most recently, we have reported tri-functional probes to monitor reversible oxidation of protein tyrosine phosphatases (PTPs) composed of a warhead bearing the cyclic β-diketone group, a binding module that targets the probe to the PTP active site, and an azide chemical reporter (**11**, **12**; Fig. 1C) (Leonard et al., 2011).

The Basic Protocols present a series of methods utilizing probes for in vitro detection of sulfenyl modifications within recombinant purified proteins (see Basic Protocol 1) and in situ detection in cultured mammalian cells (see Basic Protocols 2 and 3). Labeled sulfenylated proteins can then be visualized by either immunoblot analysis (see Basic Protocol 4) or in-gel fluorescence (see Basic Protocol 5) after being coupled to a biotin or fluorescent tag. Overall, the general approach provides a facile method for profiling sulfenyl modifications in proteins.

Ongoing development of chemical tools for selective detection of protein sulfenylation and other cysteine "oxoforms" is crucial for the progression of this rapidly growing area of research. While it is known that cysteine residues are the primary target of H_2O_2 (Paulsen and Carroll, 2010), and that sulfenylation can lead to dramatic changes in protein function, the knowledge regarding individual sites, extent, or dynamics of these modifications in cells is still in an early phase. Acquiring such information is a key step in delineating the molecular mechanisms that underlie redox-dependent control of
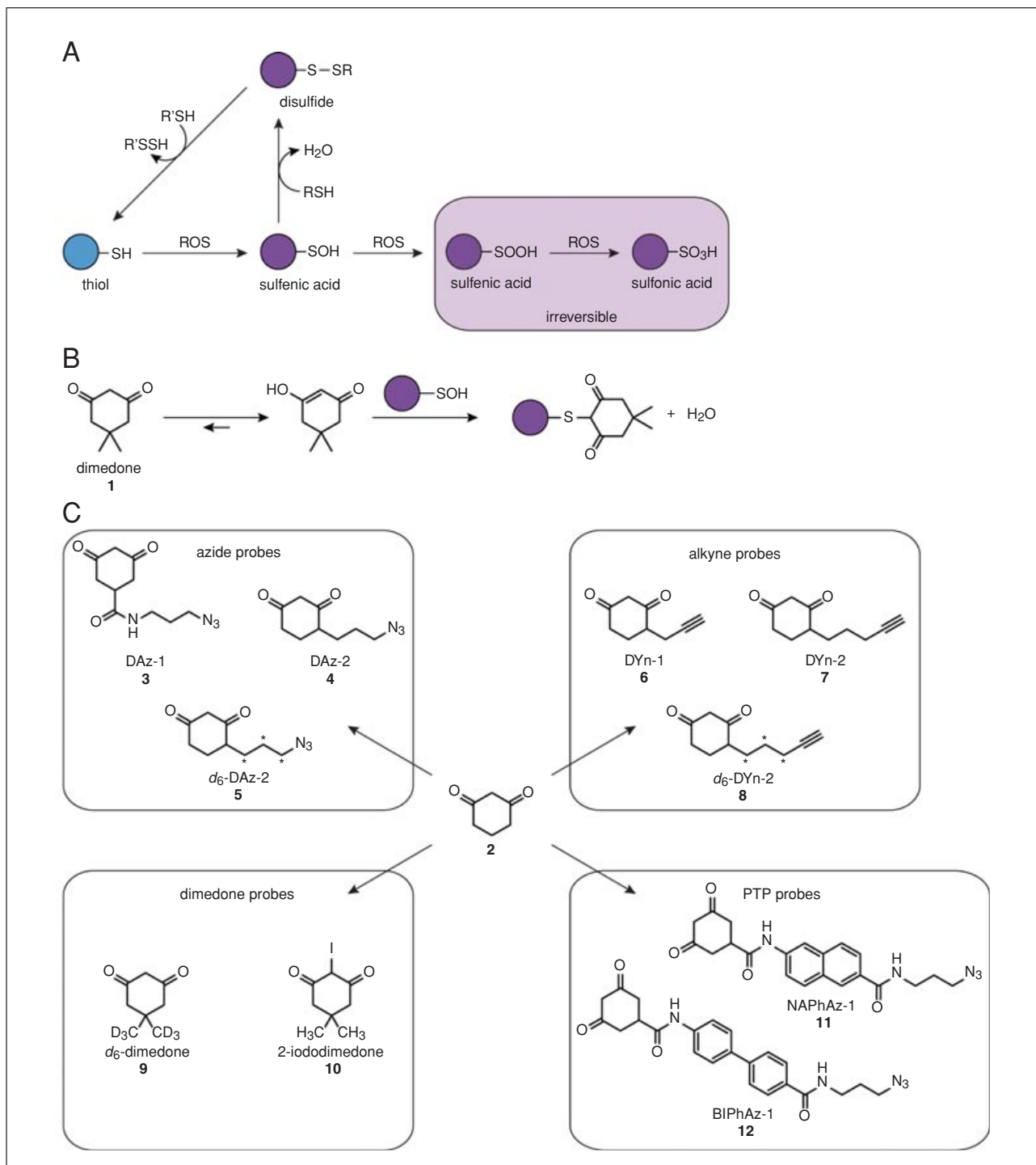
Figure 1 Bioorthogonal detection of protein cysteine oxidation. (**A**) Oxidative modifications of protein cysteines. Low pK_a thiols susceptible to oxidation can react with H_2O_2 to form a sulfenic acid, also known as sulfenylation. This modification may be stabilized by the protein microenvironment or condense with a second thiol to form an intra- or inter-molecular disulfide. Alternatively, the sulfenic acid can undergo further oxidation to the sulfinic or sulfonic acid under conditions of high oxidative stress, typically associated with disease states. (**B**) Chemoselective reaction between sulfenic acid and 5,5-dimethyl-1,3-cyclohexanedione (dimedone, **1**). (**C**) Selective probes for detecting protein sulfenic acids based on the 1,3-cyclohexanedione scaffold (**2**). These probes are functionalized with azide (**3-5**) or alkyne (**6-8**) chemical reporter groups, allow for relative quantification of sulfenic acids (**9-10**), and can target specific classes of redox-regulated proteins, such as protein tyrosine phosphatases (PTPs) (**11-12**).

intracellular signaling networks in physiology and disease. Furthermore, the exploitation of redox-modulating strategies for cancer therapy requires a detailed understanding of the structure-activity relationships of sulfenyl modifications and their roles in complex biological processes.

STRATEGIC PLANNING

Labeling Sulfenylated Proteins In Vitro

In Basic Protocol 1, the in vitro labeling method for a purified protein is demonstrated using the commercially available enzyme glyceraldehyde 3-phosphate dehydrogenase (GADPH). Upon exposure to H_2O_2, GAPDH becomes sulfenylated at the active-site cysteine (C149) (Benitez and Allison, 1974) and the modification is readily detected by the probes (Leonard et al., 2009; Seo and Carroll, 2009). This method can also be applied to investigate oxidation in other proteins that harbor H_2O_2-sensitive cysteine residues. The experimental parameters for oxidation and protein labeling described in Basic Protocol 1 have been optimized for GAPDH. Nevertheless, depending on the properties of the protein under study, it may be necessary to adjust some of the conditions if the reported procedure does not yield satisfactory results. For example, the H_2O_2 dose and exposure time can be adjusted to modulate the extent of cysteine oxidation. Similarly, probe concentration or labeling time can be changed to optimize the signal-to-noise ratio. For reference, the reaction rate of dimedone-based probes with protein sulfenic acids is on the order of 10^3 M^{-1} min^{-1} (Poole et al., 2007). It is recommended that the user first attempt the conditions outlined in Basic Protocol 1 with the protein of interest prior to adjusting any experimental conditions. If the candidate protein demonstrates H_2O_2 sensitivity, downstream experiments may involve site-directed mutagenesis of the target cysteine and/or mass spectrometry (MS) analysis of intact and digested protein after probe labeling to map the site(s) of modification. In particular, the authors' laboratory has demonstrated this with respect to wild-type and C36S Gpx3 with DAz-1 (Paulsen and Carroll, 2009) and with isotope-coded probe pairs (Seo and Carroll, 2011; Truong et al., 2011).

Labeling Sulfenylated Proteins in Living Cells

An approach for detecting protein sulfenyl modifications in situ for two human cell lines, A431 and HepG2 (Fig. 2A), is presented in Basic Protocol 2. A431 epithelial cells are a rich model for studying the biology of epidermal growth factor receptor (EGFR) signaling and for preclinical evaluation of EGFR therapeutics. The HepG2 epithelial cell line is also a suitable model system for the study of receptor-mediated H_2O_2 production and concomitant protein sulfenylation. In Basic Protocol 2, it is demonstrated that EGF stimulation of A431 cells leads to dynamic global changes in protein sulfenylation, which may be important for growth factor signaling. The protocol can be adapted to monitor changes in sulfenylation with other cell lines and stimulants, but the dose- and time-dependent effects of a particular stimulant on protein sulfenylation should be evaluated for each new cell line. Likewise, probe concentration and labeling time may require some optimization. In addition, serum starvation of cells may be required to reduce high basal levels of sulfenylation. The procedure outlined in Basic Protocol 2 is somewhat involved and may prove difficult for investigators new to mammalian cell culture. Consequently, in Basic Protocol 3, a scaled-down method is presented for detecting protein sulfenylation in HepG2 cells exposed to exogenous H_2O_2. HeLa cells may also be used in Basic Protocol 3, as they are another robust cell line that is easily propagated in the laboratory. Basic Protocols 2 and 3 call for the preparation of a single cell suspension from adherent culture for probe labeling, so it is prudent to work efficiently and avoid significant time delay after stimulation. As labeling in suspension may not prove ideal for some cell lines or stimulants, alternative methods are provided for labeling cells that remain attached to the plate in Alternate Protocols 1 and 2.
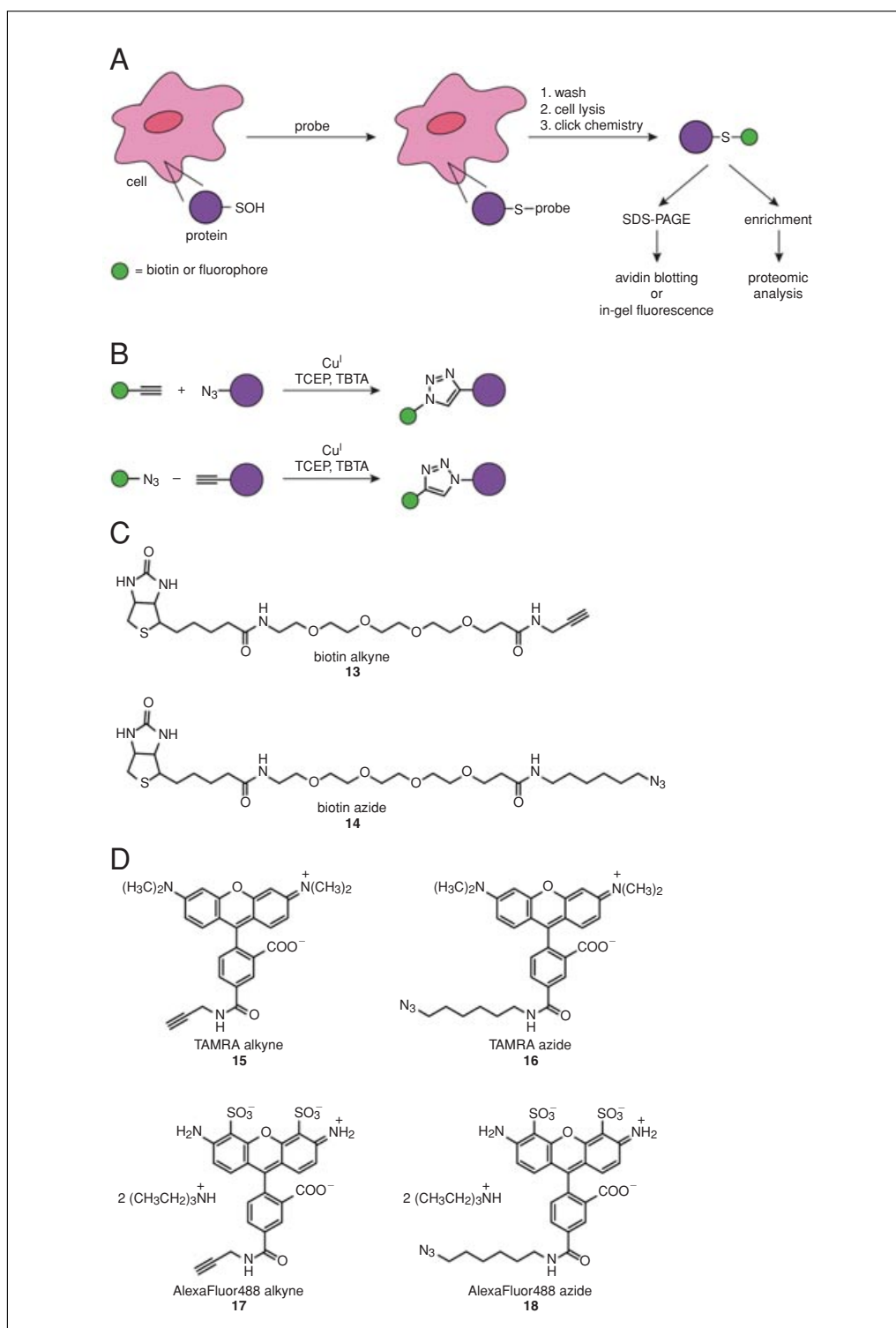
Figure 2   Cell-based detection of protein sulfenylation. (**A**) Strategy to detect protein sulfenylation in living cells. Sulfenic acids are labeled in situ using selective, cell-permeable chemical probes. Cells are then washed, homogenized, and probe-labeled proteins are conjugated to a biotin or fluorescent tag via the bioorthogonal click chemistry reaction. This approach enables downstream detection by immunoblot or in-gel fluorescence. Alternatively, biotinylated proteins may be enriched for proteomic analysis. (**B**) Generic scheme for click chemistry bioconjugation. (**C**) Biotin tags utilized in this study. (**D**) Fluorescent tags utilized in this study.

Choice of Chemical Reporter for Analyzing Protein Sulfenylation

DAz-2 and DYn-2 (**4** and **7**; Fig. 1C) can both be used to detect protein sulfenylation directly in cells. The major difference between these two probes is the identity of the chemical reporter group (azide or alkyne), which dictates the orientation of the triazole when "clicked" to the appropriate reporter tag (Fig. 2B). The choice of probe depends on downstream analysis methods. If immunoblot detection is desired, either probe can be utilized with the corresponding biotin reporter tag (**13** or **14**; Fig. 2C). However, for in-gel fluorescent detection, the exclusive use of the DYn-2 probe and azide-functionalized fluorescent tags (**16** or **18**; Fig. 2D) is recommended owing to the lower background signal reported for this orientation (Charron et al., 2009).

Bioorthogonal Ligation of Reporter-Labeled Proteins

Once proteins are labeled with DAz-2 or DYn-2, they can be detected via bioorthogonal ligation with a biotin or fluorescent tag (Fig. 2B). In the protocol, click chemistry is described as the main method of bioorthogonal ligation. The utility of click chemistry in vitro and in cells has been demonstrated by the authors and others (Speers et al., 2003; Paulsen et al., 2011; Truong et al., 2011; Wilson et al., 2011). It is also possible to detect azide-tagged proteins through Staudinger ligation with various phosphine-based reactive partners (Reddie et al., 2008; Leonard et al., 2009), although this approach is not included here.

Additional Considerations and Controls

For labeling sulfenylated proteins in vitro or in live cells, it is important to consider factors that can influence basal levels of cysteine oxidation. For example, reactive cysteines in recombinant proteins can undergo oxidation in the presence of oxygen and trace amounts of transition metal ions in phosphate or Tris-based buffer solutions. If this presents an issue for the user, buffer solutions can be made anaerobic by purging with nitrogen. In addition, cells should be cultured carefully (i.e., regular schedule for splitting, appropriate plating density, no more than 20 passages, mycoplasma testing) in high-quality medium. Poor culturing conditions can lead to cell stress and higher basal levels of cysteine oxidation. It is also recommended that the user occasionally monitor cellular ROS levels by using carboxy-H_2DCFDA (Invitrogen) and/or a general protein marker for oxidative stress, such as an antibody against the hyper-oxidized form of peroxiredoxin (Prx-SO$_3$, Abcam). Furthermore, analogous to phosphorylation studies, serum starvation may be required to reduce high basal levels of protein sulfenylation. Lastly, control reactions should always be performed in the absence of chemical probe, as indicated in each section below.

LABELING SULFENYLATED PROTEINS IN VITRO

The following is a standard protocol for in vitro labeling of sulfenyl modifications in purified proteins. GAPDH is used as the model system here because it is well known to undergo sulfenylation at its active-site cysteine (C149). This protocol can be applied to investigate sulfenylation in any other protein of interest. Proteins can be labeled with a number of biotin or fluorescent chemical reporters, allowing for immunoblot or in-gel fluorescent detection of labeled proteins.

Materials

    GAPDH, lyophilized powder (Sigma-Aldrich) or other purified protein of interest
    Tris labeling buffer (see recipe)
    50 mM tris(2-carboxyethyl) phosphine hydrochloride (TCEP; Sigma-Aldrich), prepared fresh in water

Bio-Spin 6 columns, pre-packed in Tris buffer (BioRad)
DMSO (vehicle; Sigma-Aldrich)
25 mM DAz-2 (Cayman Chemicals) *or* DYn-2 (Cayman Chemicals), prepared in DMSO
1 mM H_2O_2 stock (Sigma-Aldrich), prepared fresh in water and maintained on ice
Click labeling buffer (see recipe)
5 mM biotin tag (biotin alkyne or azide; Invitrogen) in DMSO *or* 5 mM fluorescent tag (TAMRA or AlexaFluor488 azide; Invitrogen) in DMSO
2 mM tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl] amine (TBTA; Sigma Aldrich), prepared in 4:1 DMSO/*t*-butanol (TBTA can also be synthesized by published methods; Chan et al., 2004)
50 mM $CuSO_4$, prepared fresh in water
1× PBS (Boston BioProducts)
2× Laemmli sample buffer with 10% β-mercaptoethanol (BioRad)
Mini-Protean TGX 4% to 15% Tris-Glycine protein gels (BioRad)

Centrifuge
NanoDrop2000c spectrophotometer (Thermo Scientific)
37°C incubator with shaker
Platform shaker
95°C heating block

1. Resuspend GAPDH to 4 mg/ml in Tris labeling buffer.

    GAPDH at 4 mg/ml is recommended to obtain a starting concentration of ∼110 μM. If using a different protein, the protein of interest should have a starting concentration of ∼100 to 110 μM. The relatively high protein concentration facilitates downstream gel-based MS analysis. However, if this is not desired, lower initial protein concentrations may also be used.

2. Reduce GAPDH with 1 mM TCEP for 30 min at room temperature.

    A freshly prepared stock solution of 50 mM TCEP should be used to obtain a final concentration of 1 mM TCEP for reduction of protein thiols.

3. After protein reduction, remove excess TCEP with Bio-Spin 6 columns (pre-equilibrated with Tris labeling buffer). Load 100 μl GAPDH protein per column. To collect the reduced GAPDH protein, centrifuge columns 4 min at 1000 × *g*, room temperature.

    The Bio-Spin 6 columns must be pre-equilibrated with the appropriate buffer prior to use. To achieve this, centrifuge columns for 2 min at 1000 × g to remove packing buffer. Apply the new Tris labeling buffer in 500-μl aliquots. After each application of new buffer, centrifuge the column for 1 min at 1000 × g. Repeat as required. The authors perform four washes, which results in >99.9% of buffer exchanged.

4. Determine GAPDH protein concentration using a NanoDrop2000c spectrophotometer and a molar extinction coefficient of $\varepsilon_{280} = 32,890$ M^{-1} cm^{-1}.

5. Label 25 μM GAPDH with 1 mM DAz-2 or DYn-2 (from 25 mM probe stock) by incubating GAPDH with 1.5 eq. H_2O_2 (37.5 μM from 1 mM H_2O_2 stock) or H_2O (mock treatment). In addition, prepare a no-probe control. Add reaction components to make up a total volume of 100 μl in the following order:

    GAPDH
    Tris labeling buffer (to make up remaining reaction volume)
    3.75 μl 1 mM H_2O_2 (final concentration of 37.5 μM)
    4 μl 25 mM DAz-2 or DYn-2 (final concentration of 1 mM)

6. Rock labeling reactions for 1 hr at 37°C.

7. After protein labeling, remove excess probe with Bio-Spin 6 columns (pre-equilibrated with click labeling buffer) by loading 95 µl labeling reaction per column. To collect the labeled GAPDH protein, centrifuge columns for 4 min at $1000 \times g$, room temperature.

8. Detect probe-labeled proteins by orthogonal click chemistry by adding the reaction components in the following order to each sample and mix well (total volume of the click chemistry reaction should be 100 µl):

   2 µl 5 mM biotin or fluorescent reporter tag (final concentration 100 µM)
   2 µl 50 mM TCEP (final concentration 1 mM)
   5 µl 2 mM TBTA (final concentration of 100 µM)
   2 µl 50 mM CuSO$_4$ (final concentration of 1 mM)

9. Rock click chemistry reactions for 1 hr at room temperature.

   If a fluorescent reporter tag is being used (i.e., TAMRA or AlexaFluor488 azide), perform reactions in the dark. In addition, take all necessary precautions to carry out remaining steps in the dark if a fluorescent tag is used.

10. After click chemistry, remove excess reagents with Bio-Spin 6 columns (pre-equilibrated with PBS) by loading 95 µl labeling reaction per column. To collect the labeled GAPDH protein, centrifuge columns 4 min at $1000 \times g$, room temperature.

11. Add 100 µl of 2× Laemmli sample buffer (10% β-ME) to each column eluate.

    Note that the expected recovery of GADPH applied to a Bio-Spin 6 column is ∼ 80%. This parameter must be empirically determined if a protein other than GAPDH is used.

12. Boil protein samples 10 min in a 95°C heating block.

13. Resolve samples by SDS-PAGE using Mini-Protean TGX 4% to 15% Tris-Glycine gels.

    For GADPH, 200 ng protein per lane is typically loaded.

14. Analyze results with one of the following detection methods of choice.

    a. Detect by immunoblotting (see Basic Protocol 4; Fig. 3A, DAz-2/biotin alkyne; Fig. 3B, DYn-2/biotin azide).

    b. Detect by in-gel fluorescence (see Basic Protocol 5; Fig. 3C, DYn-2/TAMRA azide; Fig. 3D, DYn-2/AlexaFluor488 azide).
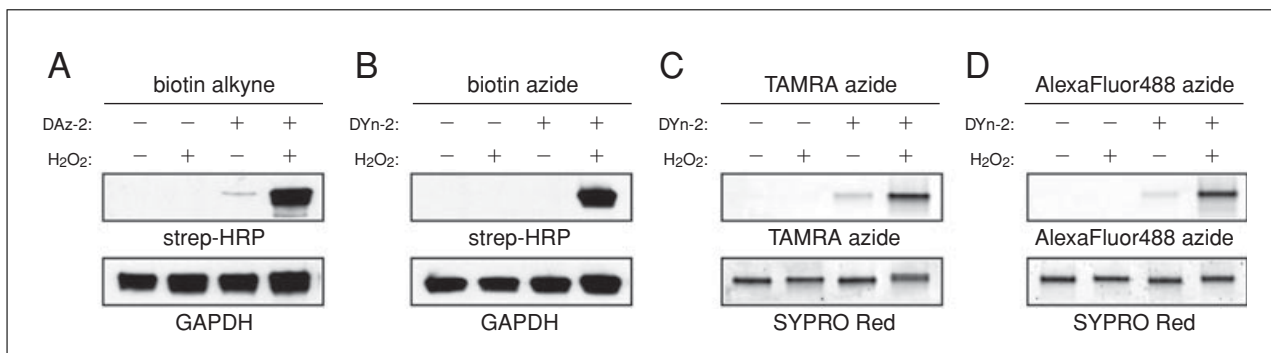

Figure 3 Detection of sulfenyl modifications with purified protein in vitro. GAPDH was stimulated with H$_2$O$_2$ and labeled with DAz-2 or DYn-2 for 1 hr. Probe-labeled GAPDH can be detected by streptavidin-HRP immunoblot (**A-B**) or in-gel fluorescence (**C-D**). Equal protein loading is demonstrated by reprobing the immunoblot with antibodies to GAPDH (immunoblot) or by SYPRO red dye staining of the SDS-PAGE gel (fluorescence).

LABELING ENDOGENOUS SULFENYLATED PROTEINS IN CELL SUSPENSION

The following is the standard protocol for labeling intact A431 cells in suspension. It has been recently demonstrated that A431 cells produce H_2O_2 upon stimulation with EGF, which leads to dynamic global changes in protein sulfenylation (Paulsen et al., 2011). In these experiments, the EGFR kinase is activated in A431 cells by stimulation with EGF ligand. It is recommended to examine other cell lines and stimulants as appropriate in specific experiments. It is also recommended that the user consider starting with Basic Protocol 3 to gain basic proficiency in these techniques prior to attempting the more involved system described in this protocol. Labeled proteins can be detected by immunoblot or in-gel fluorescence.

Materials

A431 cells (ATCC)
DMEM complete culture medium (high-glucose DMEM supplemented with 10% FBS, 1% GlutaMax, 1% MEM nonessential amino acids, and 1% penicillin-streptomycin; Invitrogen)
$1\times$ PBS (Boston BioProducts)
DMEM only (serum-free, high-glucose DMEM; Invitrogen)
30 µg/ml EGF stock (BD Biosciences), prepared in H_2O and kept on ice
0.25% trypsin (Invitrogen)
250 mM DAz-2 (Cayman Chemicals) *or* DYn-2 (Cayman Chemicals), prepared in DMSO
DMSO (vehicle; Sigma-Aldrich)
Modified RIPA lysis buffer supplemented with EDTA-free protease inhibitors and 200 U/ml catalase (see recipe)
BCA protein assay (Pierce)
5 mM biotin tag (biotin alkyne or azide; Invitrogen) in DMSO *or* 5 mM fluorescent tag (TAMRA or AlexaFluor488 azide; Invitrogen) in DMSO
50 mM TCEP (Sigma-Aldrich), prepared fresh in water
2 mM TBTA (Sigma Aldrich), prepared in 4:1 DMSO/*t*-butanol (TBTA also synthesized by published methods; Chan et al., 2004)
50 mM $CuSO_4$, prepared fresh in water
0.5 M EDTA (Boston BioProducts)
10% SDS, prepared in H_2O
$2\times$ Laemmli sample buffer with 10% β-mercaptoethanol (BioRad)
Mini-Protean TGX 4% to 15% Tris-Glycine protein gels (BioRad)

37°C incubator
Refrigerated centrifuge
1.5-ml microcentrifuge tubes
95°C heating block
Vortex

1. Grow A431 cells to 75% to 80% confluency in DMEM complete culture medium at 37°C.

    All volumes used in the remainder of this protocol are reported with respect to 100-mm dishes.

2. Remove the DMEM complete culture medium, wash plates quickly three times with enough PBS to cover cells, and incubate cells in 10 ml serum-free DMEM for 16 hr at 37°C.

    Culture medium and other cell culture reagents (e.g., 0.25% trypsin) should always be pre-warmed to 37°C prior to use in an experiment. Cells are very sensitive to changes

in temperature and all precautions should be taken to avoid sudden alterations in the culture environment.

3. After serum starvation, remove serum-free DMEM and wash plates three times with PBS.

4. Add 5 ml serum-free DMEM containing 100 ng/ml EGF to each plate of cells and stimulate for 5 min at 37°C.

   A control should be performed in parallel using un-stimulated cells to assess EGF-dependent changes in protein sulfenylation.

5. At the end of the incubation period, remove the stimulation medium and wash plates three times with PBS.

6. Lift cells with 0.25% trypsin. Add 5 ml of serum-free DMEM to quench trypsin, and collect cells by centrifuging 2 min at $1500 \times g$, room temperature.

7. Remove medium and resuspend cells in serum-free DMEM at a density of $3–4 \times 10^6$ cells/ml.

   One 100-mm plate of A431 cells will typically yield $\sim 3 \times 10^6$ cells. Accordingly, each plate is resuspended in 800 µl serum-free DMEM, and 400-µl aliquots of cell suspension are transferred to 1.5-ml microcentrifuge tubes for labeling experiments.

8. Label cells with 5 mM DAz-2 or DYn-2 (from 250 mM probe stock). To 400 µl of cell suspension sample, add 8.2 µl DAz-2 or DYn-2 and gently mix. Use DMSO as a concurrent vehicle control for all probe-labeling experiments.

9. Incubate cell labeling reactions for 1 hr at 37°C.

   After 30 min, gently mix cells. Labeling times may range from 15 min to 2 hr. This variable is cell- and stimulant-type dependent.

10. After cell labeling, remove excess probe by washing cells three times, each time with 500 µl PBS, and centrifuging 2 min at $1500 \times g$, room temperature.

    At this point, washed cell pellets can be frozen in liquid N_2 and stored at $-80°C$. Otherwise, it is recommended to proceed with the experiment up to the precipitation step.

11. Lyse cells in modified RIPA lysis buffer for 20 min on ice.

    Cell lysis can be aided by vortexing or pipetting up and down.

12. Clear lysate of cell debris by centrifuging 20 min at $16,000 \times g$, 4°C.

13. Quantify protein content of cleared cell lysate using a standard BCA assay.

    Protein concentrations typically range from 2 to 8 mg/ml, depending on the cell type.

14. If a biotin tag is used to detect labeled proteins by immunoblot, perform a pre-clear step to remove endogenous biotinylated proteins prior to click chemistry (see Support Protocol 1). If a fluorescent tag is used to detect labeled proteins by in-gel fluorescence, omit the pre-clear step and continue to the next step.

15. Transfer 100 µg cell lysate to 1.5-ml microcentrifuge tubes for each sample.

16. Detect probe-labeled proteins via click chemistry by adding the reaction components in the following order to each sample and mixing well (the total volume of the click chemistry reaction should be 100 µl):

    89 µl cell lysate (100 µg)
    2 µl 5 mM biotin or fluorescent reporter tag (final concentration 100 µM)
    2 µl 50 mM TCEP (final concentration 1 mM)

5 µl 2 mM TBTA (final concentration 100 µM)

2 µl 50 mM CuSO$_4$ (final concentration 1 mM)

Make up any remaining volume with the modified RIPA lysis buffer.

17. Rock click chemistry reactions 1 hr at room temperature.

If a fluorescent tag is used (i.e., TAMRA or AlexaFluor488 azide), perform reactions in the dark. In addition, take all necessary precautions to carry out the remaining steps of the experiment in the dark if a fluorescent reagent is used.

18. Quench click chemistry reactions with 40 mM EDTA (from 0.5 M stock). Rock reactions 5 min at room temperature.

EDTA chelates any remaining copper, thereby quenching the click chemistry reaction.

19. Perform a methanol precipitation (see Support Protocol 2) or methanol/chloroform precipitation (see Support Protocol 3) of the protein.

Either precipitation method works well and the choice is dictated by user preference.

20. Add 20 µl of 10% SDS to dissolve protein pellets.

A bath sonicator may be used at this step to help re-solubilize the protein pellets.

21. Add 20 µl of 2× Laemmli sample buffer (10% β-ME) to each sample and mix well.

22. Boil protein samples 10 min using a 95°C heating block.

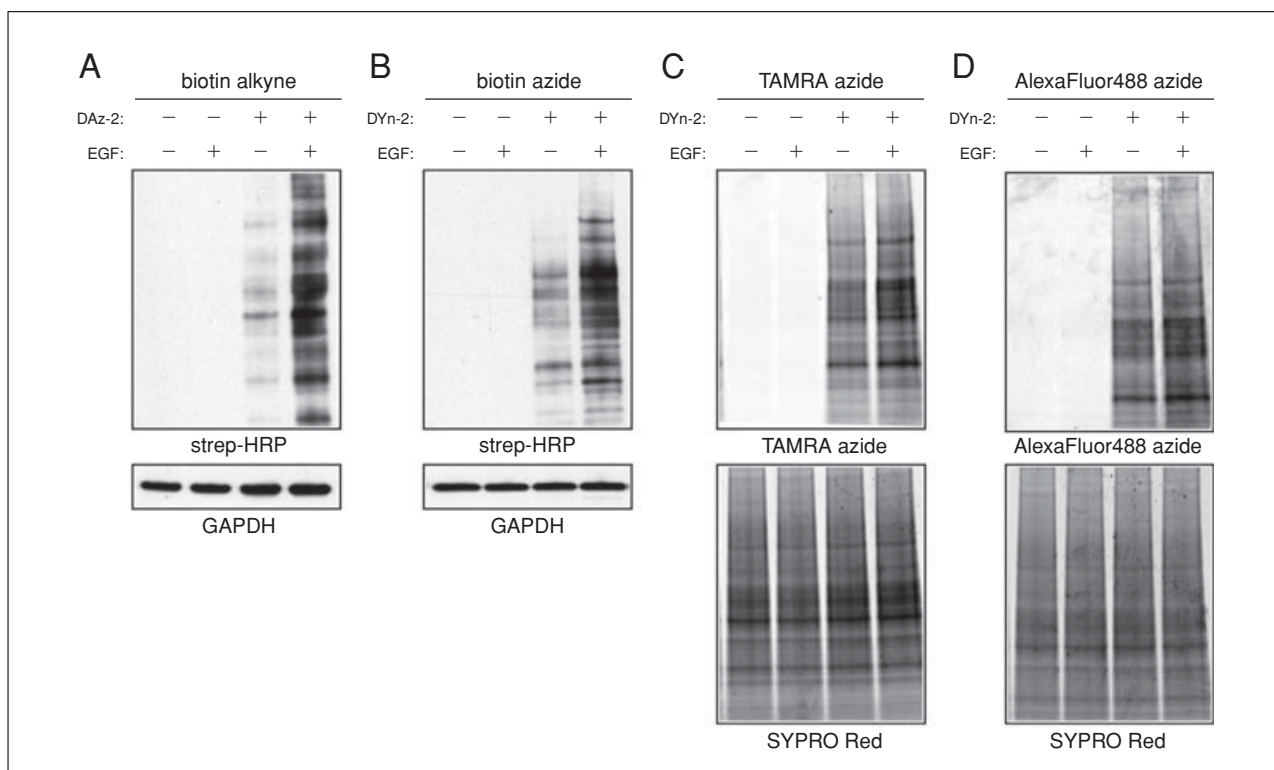23. Vortex samples and centrifuge 1 min at 16,000 × *g*, room temperature.

Figure 4 Detection of EGF-mediated protein sulfenylation in A431 cells. A431 cells were stimulated with 100 ng/ml EGF for 5 min. EGFR activation leads to the production of endogenous H$_2$O$_2$ and concomitant changes in protein sulfenylation. Cells were labeled with DAz-2 or DYn-2 for 1 hr. Sulfenylated proteins can be detected by streptavidin-HRP immunoblot (**A-B**) or in-gel fluorescence (**C-D**). Equal protein loading is demonstrated by reprobing the immunoblot with antibodies to GAPDH (immunoblot) or by SYPRO red dye staining of the SDS-PAGE gel (fluorescence).

24. Resolve samples by SDS-PAGE using Mini-Protean TGX 4% to 15% Tris-Glycine gels.

Typically, 25 μg protein/lane is adequate to detect protein sulfenylation.

25. Analyze experimental results with one of the following detection methods:

a. Immunoblotting (see Basic Protocol 4; Fig. 4A: DAz-2/biotin alkyne; Fig. 4B: DYn-2/biotin azide)

b. In-gel fluorescence (see Basic Protocol 5; Fig. 4C: DYn-2/TAMRA azide; Fig. 4D: DYn-2/AlexaFluor488 azide)

LABELING EXOGENOUS SULFENYLATED PROTEINS IN CELL SUSPENSION

The following is a standard protocol for live-cell labeling of sulfenyl modifications in HepG2 cells, which are easy to subculture and manipulate. In this protocol, exogenous H_2O_2 is applied directly to cells, as opposed to ligand stimulation of endogenous H_2O_2 production. HeLa cells can be substituted for HepG2 cells in these experiments (Leonard et al., 2009).

Materials

HepG2 cells (ATCC)
MEM complete culture medium (MEM supplemented with 10% FBS, 1% GlutaMax, 1% MEM nonessential amino acids, and 1% penicillin-streptomycin; Invitrogen)
1× PBS (Boston BioProducts)
MEM with 0.5% FBS (MEM supplemented with 0.5% FBS; Invitrogen)
100 mM H_2O_2 stock (Sigma-Aldrich), prepared fresh in water and maintained on ice
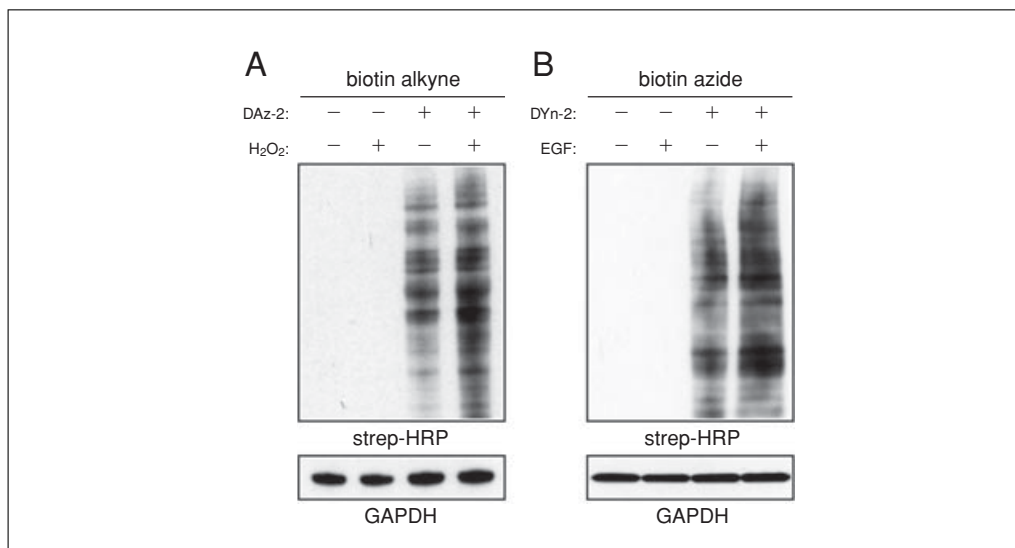
Additional reagents and equipment (see Basic Protocol 2)

Figure 5 Global changes of protein sulfenylation in HepG2 cells exposed to exogenous H_2O_2. HepG2 cells were treated with 500 μM H_2O_2 for 5 min. Cells were labeled with DAz-2 or DYn-2 for 1 hr. Sulfenylated proteins can be detected by streptavidin-HRP immunoblot (**A-B**) or in-gel fluorescence (not shown). Equal protein loading is demonstrated by reprobing the immunoblot with antibodies to GAPDH (immunoblot).

1. Grow HepG2 cells to 75% to 80% confluency in MEM complete culture medium at 37°C.

   All volumes used in this protocol are reported with respect to 100-mm dishes. Culture medium and other cell culture reagents (e.g., 0.25% trypsin) should always be pre-warmed to 37°C prior to use in an experiment.

2. Remove MEM complete culture medium, and wash plates three times with PBS.

3. Add 5 ml MEM (0.5% FBS) containing 500 μM H_2O_2 to each plate. Incubate cells with H_2O_2 for 5 min at 37°C.

   A control should be performed in parallel using untreated cells to assess H_2O_2-dependent changes in protein sulfenylation.

4. Remove MEM (0.5% FBS) containing 500 μM H_2O_2. Wash plates three times with PBS.

5. Proceed with Basic Protocol 2, steps 6 through 25.

   For HepG2 cells, take care to use MEM (0.5% FBS) medium throughout the remainder of the protocol.

   Expected results for HepG2 cells can be found in Figure 5A (DAz-2/biotin alkyne) and Figure 5B (DYn-2/biotin azide).

IMMUNOBLOT DETECTION OF BIOTINYLATED PROTEINS

The following is a standard protocol for immunoblot (western blot) detection of biotinylated proteins. After completing Basic Protocol 1, 2, or 3, the reader should refer to this protocol if a biotin tag (i.e., biotin alkyne or azide) was employed during the click chemistry step.

Materials

SDS-PAGE gel with resolved samples
PVDF membrane (0.2-μm; BioRad)
3% BSA (Fisher), prepared in TBST
TBST (Boston BioProducts)
Streptavidin-HRP antibody (GE-Healthcare)
ECL Plus western blot detection system (GE Healthcare)
GAPDH antibody (Santa Cruz Biotechnology)
Rabbit anti-mouse IgG-HRP (Invitrogen)
X-ray film

1. Transfer SDS-PAGE gel with samples of interest onto a PVDF membrane using standard immunoblot transfer techniques (Gallagher et al., 2008).

2. After transfer, block the PVDF membrane with 3% BSA for 1 hr at room temperature.

3. Wash the membrane two times with TBST for 10 min at room temperature.

4. Incubate the membrane with streptavidin-HRP (1:80,000) for 1 hr at room temperature.

   All antibodies should be diluted in TBST.

5. After antibody incubation, wash the membrane two times with TBST for 10 min.

6. Develop the membrane with chemiluminescence using the ECL Plus western blot detection system following the manufacturer's instructions.

7. Image results by film.

8. After imaging, re-block the membrane with 3% BSA for 1 hr at room temperature.

9. Wash the membrane two times with TBST for 10 min.

10. Incubate the membrane with GADPH primary antibody (1:200) for 1 hr at room temperature.

    GAPDH serves as a loading control. Other protein standards may be used, as necessary.

11. After primary antibody incubation, wash the membrane two times with TBST for 10 min.

12. Incubate the membrane with rabbit anti-mouse HRP (1:50,000) for 1 hr at room temperature.

13. After secondary antibody incubation, wash the membrane two times with TBST for 10 min.

14. Develop the membrane with chemiluminescence using the ECL Plus western blot detection system following the manufacturer's instructions.

15. Image results by film.

IN-GEL DETECTION OF FLUOROPHORE-TAGGED PROTEINS

The following is a standard protocol for in-gel detection of fluorophore-tagged proteins. After completing Basic Protocol 1, 2, or 3, the reader should refer to this protocol if a fluorescent tag (i.e., TAMRA or AlexaFluor488 azide) was used during the click chemistry step.

Materials

    SDS-PAGE gel with resolved samples
    Destain solution (see recipe)
    SYPRO ruby protein stain (BioRad)
    Wash solution (see recipe)

    Platform rocker
    Fluorescence gel scanner (e.g., Amersham Biosciences Typhoon 9400 variable
        mode imager)

1. Destain SDS-PAGE gel with the samples of interest two times with destain solution for 10 min with rocking.

    This step is necessary to remove any background fluorescence from excess reagents. Gels should be handled carefully during all destain and wash steps to avoid background smudging.

2. Wash gel with water for 10 min with rocking.

3. Scan gel using a fluorescence gel scanner.

    The authors use a Typhoon 9400 variable mode imager (Amersham Biosciences) for in-gel fluorescence analysis. The fluorescent reagents used in this protocol have the following excitation and emission wavelengths: TAMRA azide, 555/580 nm (ex/em); AlexaFluor488 azide, 495/519 nm (ex/em).

4. After visualizing results from the fluorescent reagent of choice, soak gel in destain solution for 30 min.

5. Stain gel with SYPRO ruby protein stain to demonstrate equal protein loading according to manufacturer's instructions.

    The SYPRO ruby signal can typically be seen after a 3-hr incubation.

6. After staining, wash gel two times with wash solution for 10 min, and one time with water for 10 min prior to imaging.

   SYPRO ruby has the following excitation and emission wavelengths: 280 or 450/610 nm (ex/em).

PRE-CLEARING CELL LYSATES OF ENDOGENOUS BIOTINYLATED PROTEINS

The following is a standard protocol to pre-clear cell lysates of endogenous biotinylated proteins prior to the click chemistry step.

Materials

NeutrAvidin agarose resin (Pierce)
Modified RIPA buffer (see recipe)
Cell lysates

1.5-ml microcentrifuge tubes
Centrifuge
Platform rocker at 4°C

1. Dispense NeutrAvidin resin into 1.5-ml microcentrifuge tubes for each sample.

   To pre-clear 100 μg cell lysate, use 50 μl of NeutrAvidin resin.

2. Pre-equilibrate NeutrAvidin resin with modified RIPA buffer (three times with 500 μl) for 2 min at $2300 \times g$ at room temperature.

   After the last wash, take care to completely remove modified RIPA buffer to maintain consistent protein concentration between each sample.

3. Load 100 μg cell lysate onto pre-equilibrated NeutrAvidin resin. Make up any remaining volume with modified RIPA lysis buffer.

   The click chemistry reaction is the next step of the protocol, and these reactions should have a total volume of 100 μl for 100 μg cell lysate (i.e., final protein concentration = 1 mg/ml). It is important to maintain this volume during the pre-clear step and to consider the subsequent volume of added click chemistry reagents. For example, the volume of the cell lysate loaded onto the NeutrAvidin resin for the pre-clear step should not exceed 89 μl for 100 μg cell lysate, as the remaining volume (11 μl) will be the click chemistry reagents.

4. Rock pre-cleared samples for 30 min at 4°C.

5. To collect pre-cleared cell lysate, centrifuge sample 2 min at $2300 \times g$, room temperature. Transfer cell lysate to a clean 1.5-ml microcentrifuge tube. Discard NeutrAvidin resin used for the pre-clear step.

   The cell lysates are now free of endogenous biotinylated proteins. Continue with Basic Protocol 2 for A431 cells or Basic Protocol 3 for HepG2 cells.

METHANOL PRECIPITATION OF PROTEINS

The following is a standard protocol for methanol precipitation of proteins after click chemistry. Alternatively, the reader may choose to perform a methanol/chloroform (see Support Protocol 3) precipitation.

Materials

Click chemistry reaction of cell lysate
Methanol, ice-cold
Refrigerated centrifuge

1. Add 1 ml ice-cold methanol to each sample and vortex to mix.

2. Allow proteins to precipitate overnight at $-80°C$.

3. Collect protein pellet by centrifuging samples 10 min at $16,000 \times g$, $4°C$.

4. Carefully remove methanol, being careful not to disturb the protein pellet. Wash protein pellet with 1 ml ice-cold methanol.

   The second methanol wash is necessary to remove any residual click chemistry reagents.

5. Centrifuge samples 10 min at $16,000 \times g$, $4°C$.

6. Carefully remove the methanol, leaving protein pellet behind.

   Ensure all traces of excess methanol are removed prior to continuing a protocol.

METHANOL/CHLOROFORM PRECIPITATION OF PROTEINS

The following is a standard protocol for methanol/chloroform precipitation of proteins after click chemistry.

Materials

   Click chemistry reaction of cell lysate
   Methanol, ice-cold
   Chloroform, ice-cold
   Refrigerated centrifuge

1. To perform a methanol/chloroform precipitation, add the following precipitation components in order to each sample, vortexing sample after addition of each component:

   600 μl methanol
   150 μl chloroform
   400 μl H_2O

2. Centrifuge samples 10 min at $16,000 \times g$, $4°C$.

3. Carefully remove and discard as much of the upper aqueous layer as possible, without disturbing the interface layer containing the protein precipitate.

   A thin interface layer containing the precipitated protein should form between the upper aqueous layer and the lower organic layer.

4. Add 1 ml ice-cold methanol to each sample and vortex to mix.

5. Centrifuge samples 10 min at $16,000 \times g$, $4°C$.

6. Carefully remove methanol, being careful not to disturb the protein pellet. Wash the protein pellet with 1 ml ice-cold methanol.

   The second methanol wash is necessary to remove any residual click chemistry reagents.

7. Centrifuge samples 10 min at $16,000 \times g$, $4°C$.

8. Carefully remove methanol, leaving the protein pellet behind. Allow any remaining methanol or chloroform to evaporate by leaving sample tubes open on the bench. Allow protein pellet to air-dry for 30 min to 1 hr at room temperature.

   The dried protein pellet will be thin and have a translucent white color. It is crucial to allow the protein pellet to completely dry prior to proceeding with the remainder of the protocol. If it is not dry, the protein pellet will be extremely difficult to resuspend.

ON-PLATE LABELING OF ENDOGENOUS PROTEIN SULFENYLATION IN CELLS

The following is a standard protocol for labeling cells that remain attached to the plate. Although comparable results are typically observed for suspension versus plate detection of sulfenyl modifications (Figs. 4A and 6A), labeling cells in suspension may not prove ideal for some cell lines or stimulants. The reader should determine which labeling method is best for their purposes.

For materials, see Basic Protocol 2

1. Follow Basic Protocol 2, steps 1 through 5.

   For on-plate labeling, cells can be grown in 60-mm tissue culture dishes to reduce probe consumption. Media volumes should be adjusted accordingly for smaller culture plates. For example, EGF stimulation should be performed with 2 ml for 60-mm plates, rather than the 5 ml required for 100-mm plates.

2. Add 2 ml serum-free DMEM containing 5 mM DAz-2 or DYn-2 (from 250 mM probe stock) to each plate of cells.

   For 2 ml medium, 40.8 μl of 250 mM DAz-2 or DYn-2 is needed to achieve a final concentration of 5 mM. In addition, a DMSO vehicle control should always be included for unstimulated and EGF-stimulated cells.

3. Label cells for 1 hr at 37°C.

4. Remove serum-free DMEM containing 5 mM DAz-2 or DYn-2. Wash plates three times with PBS.

5. Lyse cells in modified RIPA lysis buffer using a rubber policeman. Transfer cell lysates to a clean 1.5-ml microcentrifuge tube.
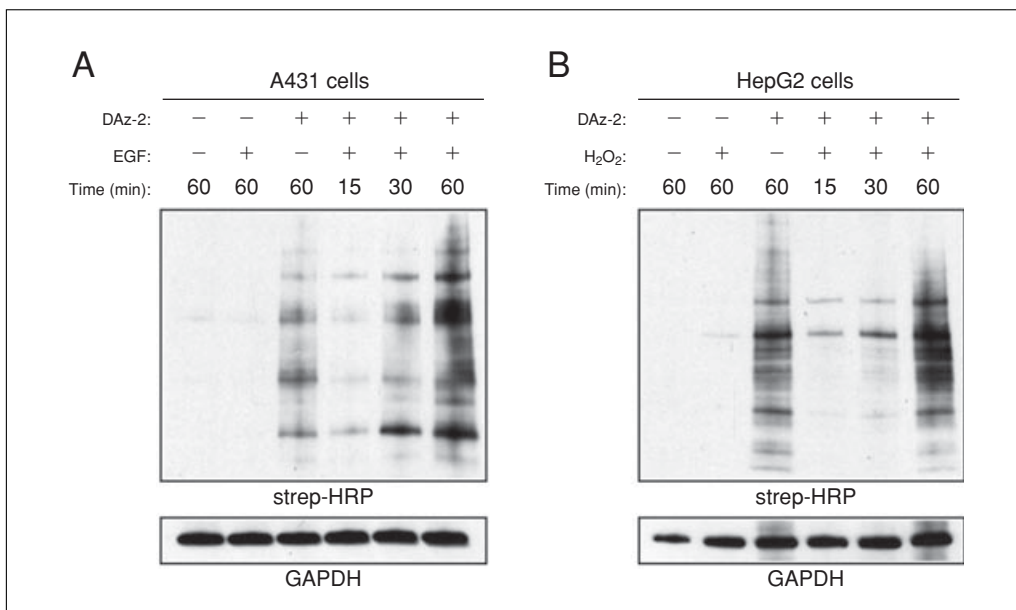

Figure 6 Labeling and detection of protein sulfenylation in adherent A431 and HepG2 cells. Cells were exposed to 100 ng/ml EGF (A431) or 500 μM H_2O_2 (HepG2) for 5 min. After treatment, cells remained attached to the tissue culture plate and were labeled with DAz-2 for the indicated times. Sulfenylated proteins were visualized by streptavidin-HRP immunoblot for (**A**) A431 cells and (**B**) HepG2 cells. Equal protein loading is demonstrated by reprobing the immunoblot with antibodies to GAPDH (immunoblot).

6. Proceed with Basic Protocol 2, steps 11 through 25.

Figure 6A shows representative results for on-plate labeling of protein sulfenyl modifications with DAz-2/biotin alkyne in A431 cells.

Time-dependent labeling of A431 cells that remain attached to the plate indicates that 1-hr labeling yields the best results.

ON-PLATE LABELING OF EXOGENOUS PROTEIN SULFENYLATION IN CELLS

The following is a standard protocol for on-plate labeling of sulfenylated proteins in HepG2 cells.

For materials, see Basic Protocol 3.

1. Follow Basic Protocol 3, step 1 through 4.

For on-plate labeling, cells can be grown in 60-mm tissue culture dishes to reduce probe consumption. Media volumes should be adjusted accordingly for smaller culture plates. For example, the H_2O_2 stimulation should be performed with 2 ml for 60-mm plates, rather than the 5 ml used for 100-mm plates.

2. Add 2 ml MEM (0.5% FBS) containing 5 mM DAz-2 or DYn-2 (from 250 mM probe stock) to each plate of cells.

For 2 ml medium, 40.8 µl of 250 mM DAz-2 or DYn-2 is needed to achieve a final concentration of 5 mM. In addition, a DMSO control should always be included for untreated and H_2O_2-exposed cells.

3. Label cells for 1 hr at 37°C.

4. Remove MEM (0.5% FBS) containing 5 mM DAz-2 or DYn-2. Wash plates three times with PBS.

5. Lyse cells in modified RIPA lysis buffer using a rubber policeman. Transfer cell lysate to a clean 1.5-ml microcentrifuge tube.

6. Proceed with Basic Protocol 2, steps 11 through 25.

Figure 6B depicts representative results for on-plate labeling of HepG2 cells with DAz-2/biotin alkyne.

Time-dependent labeling of HepG2 cells that remain attached to the plate indicates that 1-hr labeling yields the best results.

REAGENTS AND SOLUTIONS
Use Milli-Q purified water in all recipes and protocol steps.

Click labeling buffer, pH 7.4

50 mM triethanolamine, pH 7.4
1% SDS
Filter sterilize using a 0.2-µm filter
Store up to 6 months at room temperature

Destain solution

40% methanol
50% H_2O
10% acetic acid

Mix 400 ml methanol, 500 ml H_2O, and 100 ml acetic acid to make 1 liter destain solution. Store up to 1 year at room temperature.

Modified RIPA lysis buffer, pH 7.4

50 mM triethanolamine, pH 7.4
150 mM NaCl
1% NP-40
1% sodium deoxycholate
0.1% SDS
Filter sterilize using a 0.2-µm filter
Store up to 1 month at room temperature

Shortly before use, add EDTA-free complete mini protease inhibitors (Roche) according to manufacturer's instructions and 200 U/ml catalase (Sigma-Aldrich).

Tris labeling buffer, pH 7.4

50 mM Tris·Cl, pH 7.4
150 mM NaCl
Filter sterilize using a 0.2-µm filter
Store up to 6 months at room temperature

Wash solution

10% methanol
70% H_2O
10% acetic acid

Mix 100 ml methanol, 700 ml H_2O, and 100 ml acetic acid to make 1 liter wash solution. Store up to 1 year at room temperature.

COMMENTARY

Background Information

Historically, methods to detect reactive cysteines and H_2O_2-mediated oxidation of protein thiols have required the homogenization of cells, which disrupts the native environment. Under these conditions, cellular redox balance and protein structure are compromised, leading to artifactual oxidation during sample processing. This core issue is typically ignored in redox proteomic studies, but casts a pall on the biological significance of such data. To address this problem, small-molecule probes that enable sulfenylated proteins to be trapped and tagged directly in cells have been developed. Using this new approach, the molecular mechanism of H_2O_2-sensing in yeast has been dissected (Paulsen and Carroll, 2009), the first in situ proteomic study of protein sulfenylation in mammalian cells has been reported (Leonard et al., 2009), striking differences in sulfenylation profiles among breast cancer subtypes has been uncovered (Seo and Carroll, 2009), a bacterial protein that functions in desulfenylation has been discovered (Depuydt et al., 2009), and sulfenylation functions as a global signaling mechanism akin to phosphorylation during EGFR signaling has been demonstrated (Paulsen et al., 2011). Clearly, selective chemical reporters have opened the door to a better understanding of the important biological role of protein cysteine sulfenylation.

The first global analysis of sulfenylated proteins in HeLa cells revealed that potentially modulatory cysteine residues exist in almost 200 individual proteins, including kinases, phosphatases, transporters, GTPases, and transcription factors, thereby extending this form of redox regulation to a wide range of biological activities (Leonard et al., 2009). Nonetheless, the vast majority of H_2O_2-sensitive protein cysteine residues await identification. This is a vigorous area of research in the authors' laboratory and, towards this end, they have developed two complementary isotope-coded probe pairs to quantify changes in protein sulfenylation (**9**, **10** and **4**, **5**; Fig. 1C) (Seo and Carroll, 2011; Truong et al., 2011). Most recently, the authors have reported reagents to selectively monitor reversible oxidation of PTPs by adding an active site-targeting module to the sulfenyl probes (**10**, **11**; Fig. 1c) (Leonard et al., 2011).

Critical Parameters and Troubleshooting

The order in which reagents are added during experiments to purified protein in vitro or cells is an important consideration. In general, the ligand stimulant (or H_2O_2) is added before the probe-labeling step. However, this parameter should be investigated on a case-by-case basis, in addition to dose- and time-dependent changes in labeling.

For cell labeling experiments, cells should be cultured carefully without sudden changes in the culture environment. For example, culture reagents such as media and trypsin should be pre-warmed to $37°C$ prior to use. Depending on the cell line, culture media should be replenished every 2 to 3 days. As with many biological processes studied in a cell culture model, the confluency at the time of stimulation and labeling can impact the final results. If stimulant- or H_2O_2-dependent changes in protein sulfenylation are difficult to discern, a number of factors should be examined. All stocks (ligand stimulant and probes) should always be kept on ice during the experiment or otherwise stored at $-30°C$ when not in use. In addition, lysis buffer and cell lysates should be kept on ice at all times. Once cells have been stimulated, subsequent wash and lifting steps should be carried out in a timely manner. Any unnecessary delays in sample handling prior to cell lysis should be avoided, as this may also affect final results. Additionally, for maximum probe potency, culture media used during cell labeling should not exceed 0.5% FBS.

Click chemistry reactions should always be performed with fresh TCEP and $CuSO_4$ stocks. Typically, these stocks are prepared fresh directly before setting up the reactions. TCEP can oxidize over time, which would reduce the efficiency of the reaction since it is required for reduction of $CuSO_4$ to Cu(I). In addition, the second methanol wash in both precipitation procedures is crucial to completely remove excess click chemistry reagents. Failure to do so may result in higher background signals during the visualization process. If equal loading is not observed, it is also important to ensure that the protein pellets are completely re-solubilized. In some cases, this can be a challenge, particularly for samples that have been methanol/chloroform precipitated. Protein pellets must be completely free of chloroform prior to re-solubilization, otherwise they will not fully dissolve.

For in-gel fluorescence experiments, special precautions should be taken once the fluorescent tag is added to the sample. Steps such as the click chemistry reaction, SDS-PAGE analysis, and destaining should be performed in the dark to avoid sample bleaching. During SDS-PAGE analysis, the dye front should be run completely off the gel during electrophoresis to remove any unincorporated fluorescent tag and minimize background fluorescence. Gels should also be thoroughly destained and washed prior to imaging. In addition, only clean gloves and containers should be used to handle the gels. Because in-gel fluorescence is an extremely sensitive technique, faulty gel handling can lead to fingerprints and smudges during the visualization process. Gels should only be handled on the edges as carefully as possible with gloved hands, to avoid undesirable blemishes on the scanned image.

Anticipated Results

Anticipated results for labeling of sulfenic acids in a purified protein (GAPDH) can be found in Figure 3 for both immunoblot (Fig. 3A,B) and in-gel fluorescence detection (Fig. 3C,D). It is expected that the active site cysteine should exhibit a robust H_2O_2-dependent increase in probe labeling. If a peroxide-dependent effect is not observed in the protein of interest, it is possible that the cysteine(s) are not H_2O_2-sensitive or that the basal level of oxidation is high. Similarly, anticipated results for labeling of protein sulfenylation in cells (A431) with a stimulant (EGF) that leads to endogenous production of H_2O_2 can be found in Figures 4 and 6 for immunoblot (Figs. 4A,B and 6A) and in-gel fluorescence detection (Fig. 4C,D). If a stimulant induces endogenous H_2O_2 production, an increase in sulfenylated proteins should be observed. Finally, a protocol for cells (HepG2) stimulated with H_2O_2 is provided so that the user may develop and hone their skills as necessary (Figs. 5A,B and 6B). DMSO vehicle control lanes should have negligible background signal. In cell lysate samples that have been precleared with NeutrAvidin agarose resin, signal may still be evident at $\sim$66 and 97 kDa. In fact, these bands may be observed for unlabeled and probe-labeled samples. This is not cause for major concern and indicates that biotinylated proteins could not be completely depleted from samples. Results can be considered successful if the signal from probe-labeled samples is higher than the control lanes, and if a stimulant-dependent increase of signal is observed. If the user finds it difficult to observe the desired stimulant-dependent response, a number of solutions can be explored. Simple adjustments such as changing the

concentration of the probe, stimulant, labeling times, and protein loading should be considered (see Strategic Planning or Critical Parameters and Troubleshooting for more detailed explanations). The sensitivity of the detection method can also be a factor and thus, immunoblot and in-gel fluorescence should both be explored.

Time Considerations

The experiments outlined in this protocol typically require between 2 and 3 days from labeling to detection of sulfenylated proteins. Experiments with purified protein in vitro (Basic Protocol 1) can be paused after sample buffer has been added to click chemistry reactions (6 to 8 hr). Immunoblot or in-gel fluorescence detection can be carried out on the same or following day, depending on the user. Live-cell experiments (Basic Protocols 2 and 3) can be paused at the methanol or methanol/chloroform precipitation step (6 to 8 hr). For live-cell experiments, detection of sulfenylated proteins should be performed on the next day after samples have been resuspended. Additionally, the user may pause the experiment directly after the cells are labeled with DAz-2 or DYn-2. After washing cells to remove labeling medium, cell pellets can be harvested and stored at $-80°C$ (see Basic Protocol 2, step 10). However, after cell lysis, it is recommended that the experiment be continued to the precipitation step to avoid compromising the click chemistry reaction. Basic Protocol 2 can be adapted for other cell types, but each system will require optimization.

Acknowledgements

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