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Measurement of Protein Sulphydryls in Response to Cellular Oxidative Stress Using Gel Electrophoresis and Multiplexed Fluorescent Imaging Analysis

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Abstract

The significance of free radicals in biology has been established by numerous investigations spanning a period of over 40 years. Whereas there are many intracellular targets for these radical species, the importance of cysteine thiol posttranslational modification has received considerable attention. The current studies present a highly sensitive method for measurement of the posttranslational modification of protein thiols. This method is based on labeling of proteins with monofunctional maleimide dyes followed by 2D gel electrophoresis to separate proteins and multiplexed fluorescent imaging analysis. The method correctly interrogates the thiol/disulfide ratio present in commercially available proteins. Exposure of pulmonary airway epithelial cells to high concentrations of menadione or *t*-butyl hydroperoxide resulted in the modification of cysteines in more than 141 proteins of which 60 were subsequently identified by MALDI-TOF/TOF MS. Although some proteins were modified similarly by these two oxidants, several showed detectably different maleimide ratios in response to these two agents. Proteins that were modified by one or both oxidants include those involved in transcription, protein synthesis and folding, and cell death/growth. In conclusion, these studies provide a novel procedure for measuring the redox status of cysteine thiols on individual proteins with a clearly demonstrated applicability to interactions of chemicals with pulmonary epithelial cells.

Introduction

Over the past 40 years, the production and control of free radicals in the cellular milieu has been the subject of intense interest among the toxicology community. High concentrations of free radicals, generated from a number of toxicants interact with lipids, proteins, and DNA to produce a host of downstream events, leading to responses that vary from cytotoxicity to cancer (for review see ^{1, 2}). More recently, the role of these oxidants as intracellular signaling molecules and the subsequent importance of posttranslational modification of cysteine sulphydryls in mediating a host of diseases associated with oxidative stress have been recognized (^{3–8}). These diseases include Parkinson's, diabetes, cancer, arthritis, parenchymal lung diseases, and many others. The essential nature of cysteines in protein function and the nucleophilic properties of thiols, some of which can be highly reactive toward both oxidants and electrophiles, support the need to understand the overall role of cysteine thiol loss in specific proteins as a mechanism for alteration of cellular homeostasis.¹

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Reactive cysteines are contained within the active site of proteins, and modification of these cysteines produces demonstrable changes in protein function (reviewed in ⁹). For example, changes in the redox status of Keap1 are essential for the induction of phase 2 and antioxidant genes (10⁻¹²). However, not all posttranslational sulfhydryl modification results in an alteration of protein function. For example, studies by Dalle-Donne et al. (13) have demonstrated that facile modification of Cys-374 of actin by acrolein does not dramatically influence polymerization of this structural protein. Rather, this residue appears to be preferentially modified and only when Cys-374 is fully saturated does acrolein interact with histidines, which subsequently results in changes in the kinetics of actin polymerization.

Antioxidant enzymes including thioredoxins, peroxiredoxins, and glutaredoxins are essential in maintaining cellular thiol redox balance. These proteins are markedly upregulated by oxidative stress (14) and all contain active-site thiols, which can become oxidized (15). In the case of some of the peroxiredoxins, active-site thiols become oxidized, resulting in protection of other sensitive cellular thiol containing biomolecules (16). This finding is consistent with the observation that overexpression of peroxiredoxin 6 in transgenic mice increased cellular resistance to lung injury from hyperoxia (17). These findings also are consistent with work showing marked decreases in peroxiredoxin 6 activity associated with the interaction of reactive metabolites of butylated hydroxytoluene (quinone methide) with this protein (18). Thus, there is substantial evidence to support the contention that modification of critical protein thiols, either through covalent adduct formation or through oxidation, is capable of producing highly deleterious effects on cellular homeostasis. However, a complete understanding of the “critical” proteins and the degree to which they must be oxidized prior to initiation of the series of events that leads to cellular injury is currently unknown. Accordingly, methods for examining thiol loss, either through oxidation or adduct formation, for both soluble thiols (mainly GSH) and cysteines in individual proteins in the proteome are needed to more completely address these issues.

Several methods are available for measuring protein thiols, and these have been reviewed comprehensively (19⁻²¹). Most depend upon alkylation of protein thiols with labels that have either chromophores or are radioactive; older methods were intended primarily for measuring reduced protein thiols (22⁻²⁴). Some of the more recent methods, intended for use with mass spectrometry, use thiol-reactive ICAT labels. In many cases, accessible protein thiols are derivatized, followed by reduction of S-nitrosothiols with ascorbate, sulfenic acids with arsenite, and disulfides with DTT. The biotin switch technique has been shown to provide good quantitative determination of nitrosylated proteins (25). With the exception of the approaches based on detection and quantitative measurements by mass spectrometry, these differential labeling techniques are not particularly quantitative because samples derived from free thiol labeling must be processed separately from those where disulfide reduction is followed by labeling.

The current study extends earlier work on the labeling of purified proteins with fluorescent maleimides (26) and presents multiplexed fluorescence methods that are useful in measuring thiol loss during cellular exposure to electrophiles/oxidants. The method has been validated with commercially available proteins and is used to assess the oxidation of airway epithelial cell proteins recovered from *in situ* incubations with menadione or *t*-butyl hydroperoxide, two strong, model oxidizing reagents.

¹Abbreviations: BSA, bovine serum albumin; NDSB-195, nondetergent sulfobetaine-195; TBL, Tris buffered lysis solution; TBP, tributylphosphine; DTNB, dithiobisnitrobenzoic acid; NT-TBL, nonthiourea lysis solution; PI, protease inhibitor.

Experimental Procedures

Reagents

Bovine serum albumin (BSA), trypsinogen, aldehyde dehydrogenase, tributylphosphine, *t*-butyl hydroperoxide, and menadione (2-methyl-1,4-naphthoquinone) were obtained from Sigma Chemical Co (St. Louis, MO). Protease inhibitor cocktail III, nondetergent sulfobetaine-195 (NDSB-195), benzamidine, 1,10-phenanthroline, aprotinin, pepstatin, and leupeptin were purchased from Calbiochem (La Jolla, CA). Rhinohide acrylamide/bisacrylamide was from Molecular Probes (Eugene, OR). Biospin 30 size exclusion columns, Precision Plus Protein Standard, and Bio-Rad Protein Assay solution were purchased from Bio-Rad (Hercules, CA). Sea Plaque low melting temperature agarose and Isogel agarose were from Cambrex BioScience Rockland, Inc. (Rockland, ME). NuPAGE 12% Bis-Tris gels were purchased from Invitrogen (Carlsbad, CA). Cy3 and Cy5 maleimide monoreactive dyes, and all other electrophoresis materials were obtained from GE Health Care (Piscataway, NJ). Please note that these dyes are not the CyDIGE maleimides but are the disulfonic acid maleimides. All solutions were prepared with deionized water (resistivity 18.1 M Ω /cm).

Animals

All animal use was approved by the Animal Use and Care Committee at the University of California, Davis. Male Sprague–Dawley rats (225–250 g) and mice (Swiss Webster, 25–30 g) were purchased from Harlan (Indianapolis, IN). Animals were allowed free access to food and water and were housed in an AAALAC accredited facility in HEPA-filtered cage racks at the University of California, Davis, for at least 5 days before use.

Maximal Cy Labeling and Measurement of Free and Disulfide Bound Thiols by 1DE

A schematic diagram outlining the Cy labeling procedure is shown in Figure 1. Purified protein stock solutions of 4 mg/mL were made containing aldehyde dehydrogenase, trypsinogen, or BSA in Tris buffered lysis solution (TBL) (2 M thiourea, 7 M urea, 4% w/v CHAPS, 0.5% w/v Triton X-100, 25 mM Tris pH 7.6, and 2% v/v protease inhibitor cocktail III). Aliquots of these purified protein stock solutions containing 3.0, 2.1, and 1.6 nmols aldehyde dehydrogenase, 3, 2.4, and 1.6 nmols trypsinogen, and 1.0, 0.81, and 0.58 nmols BSA were added to 40 nmols of Cy3 maleimide, creating solutions of aldehyde dehydrogenase at 0.063, 0.055, and 0.046 mM, trypsinogen at 0.077, 0.068, and 0.053 mM, and BSA at 0.027, 0.024, and 0.019 mM. All samples were allowed to incubate in the dark for 2 h at room temperature. The reaction of maleimide derivatives with free sulfhydryls is complete in less than 2 h (26–27). From this point onward, light exposure was minimized for all CyDye-labeled samples. Unreacted Cy3 maleimide dye was separated from the labeled samples by size exclusion chromatography on Biospin 30 columns equilibrated with TBL. The first fraction, containing approximately 500 μ L of Cy3-labeled proteins, was saved. To reduce disulfides, tributylphosphine (TBP, 40 μ L, 200 mM) was added to bring the samples to approximately 16 mM final concentration, and samples were allowed to incubate at room temperature for 30 min. Cy5 maleimide (40 nmols) was added to the Cy3-labeled protein fraction, and this solution was allowed to incubate at room temperature for a further 2 h. The Cy-labeled protein solutions were diluted with TBL solution to achieve concentrations of 4.8, 3.4, and 2.6 μ M aldehyde dehydrogenase, 4.8, 3.8, and 2.5 μ M trypsinogen, and 1.7, 1.3, and 0.93 μ M BSA. These protein solutions (10 μ L each) were loaded onto a 10 cm $\times$ 10 cm 10% Rhinohide acrylamide/bisacrylamide gel and electrophoresed at 25 mA per gel constant for approximately 1.5 h. A separate lane containing 2 μ L of Precision Plus Protein Standard was included for molecular mass markers.

Methods adapted from DiMonte et al. (23) were used to verify some of the data on free thiol levels in BSA and rat airway epithelial cell proteins obtained by lysis lavage. Glutathione was

used as a standard and absorbance at 412 nm covered the range of sample values. Briefly, cuvettes contained 2 mM 5'-dithiobis(2-nitrobenzoic acid) (DTNB) dissolved in 50 mM sodium acetate, 100 μ L 1 M Tris pH 8.0, glutathione standard or sample in a total volume of 1 mL. BSA solutions were made at 2.9, 1.4, and 0.7 mM, and 100 μ L was added to each cuvette. Concentrations of 250, 500, and 750 μ g of rat lysis lavage solution were added to respective cuvettes. Solutions in cuvettes were mixed by pipetting and read at 412 nm on a Beckman DU70 spectrophotometer after 5 min. Moles of thiol per mole of BSA were calculated using the glutathione standard curve.

Recovery of Rat Epithelial Cell Proteins by Lysis-Lavage

Epithelial cell proteins were recovered by lysis lavage from an individual rat as described previously by Wheelock et al. (28). The lungs were lavaged with nonthiourea lysis solution (NT-TBL) containing 1 M NDSB-195, 7 M urea, 4% w/v CHAPS, 0.5% w/v Triton X-100, 25 mM Tris pH 7.6, and 2% v/v protease inhibitor solution (PI) containing 26.4 mM benzamidine, 100 mM phenanthroline, 0.15 mM aprotinin, 1.46 mM pepstatin, and 2.1 mM leupeptin. The lysis lavage sample was frozen at -80°C until the following experiments could be performed.

Maleimide Hydrolysis

Three 150 μ L aliquots of rat lysis lavage sample were allowed to react with 250 nmols of Cy3. After 2 and 16 h, 50 μ L aliquots were removed from each of the three reaction tubes and immediately frozen at -80°C to halt labeling. The last 50 μ L in each of the three tubes was reduced with 6.65 μ L of 200 mM tributylphosphine (24 mM final concentration) for 30 min. All nine Cy3-labeled samples were diluted with TBL and 4 $\times$ Laemmli sample buffer to achieve a protein concentration of 0.5 μ g/ μ L. These samples (1 μ L each) were loaded onto a 10 cm $\times$ 10 cm $\times$ 1.0 mm 15-well NuPAGE 12% Bis-Tris gel and electrophoresed at 25 mA per gel constant for approximately 1.5 h. A separate lane containing 2 μ L of Precision Plus Protein Standard was included for molecular mass markers.

Determination of Maleimide Dye Labeling Bias by 1DE

Six 100 μ L aliquots of rat lysis lavage sample were allowed to react with 125 nmols each of Cy3 ($n = 3$) or Cy5 ($n = 3$) for 16 h. The Cy-labeled samples were diluted with TBL and 4 $\times$ Laemmli sample buffer to achieve a concentration of 0.5 μ g/ μ L. These samples (1 μ L each) were loaded onto a 10 cm $\times$ 10 cm $\times$ 1.0 mm 15-well NuPAGE 12% Bis-Tris gel and electrophoresed at 25 mA per gel constant current for approximately 1.5 h. A separate lane containing 2 μ L of Precision Plus Protein Standard was included for molecular mass markers.

Determination of Optimal Concentration of Tributylphosphine by 1DE

Two 75 μ L aliquots and one 150 μ L aliquot of the rat lysis lavage sample were allowed to react with 62.5 and 125 nmols of Cy3, respectively. Following the 16 h Cy3 labeling, tributylphosphine at final concentrations of 18.2, 3.92, and 0.995 mM were added to each sample respectively and allowed to react for 30 min. The second labeling was performed by adding 62.5 and 125 nmols Cy5, respectively. The reaction mixture was allowed to incubate for 2 h. The Cy-labeled samples were diluted with TBL and 4 $\times$ Laemmli sample buffer to achieve a protein concentration of 0.5 μ g/ μ L. These samples (1 μ L each) were loaded onto a 10 cm $\times$ 10 cm $\times$ 1.0 mm 15 well NuPAGE 12% Bis-Tris gel and electrophoresed at 25 mA per gel constant current for approximately 1.5 h. A separate lane containing 2 μ L of Precision Plus Protein Standard was included for molecular mass markers.

Protein Visualization and Analysis in 1DE

After electrophoresis, protein gels were fixed in 50% v/v methanol, 7% v/v acetic acid overnight in plastic storage containers wrapped in aluminum foil to prevent photobleaching of the fluorescent labels. All gels were scanned on a Typhoon 8600 Variable Mode Imaging Scanner (Cy3: 532 nm excitation, 555 nm emission filter with a 20 nm band-pass; Cy5: 633 nm excitation, 670 nm emission filter with a 30 nm band-pass) with a resolution of 100 μ m. Photomultiplier settings varied between 400 and 800 mV and were set to the highest levels possible without signal saturation. Volume analysis of the gel bands was performed with *ImageQuant* v5.1 (Molecular Dynamics, Sunnyvale, CA, now GE Health Care) and calculations were performed in *Microsoft Excel*. Statistical comparisons of spot volumes between groups were made using a One-Way ANOVA in *SigmaStat* v3.0. Differences were considered significant when $p \leq 0.05$.

Airway in situ Incubations and Recovery of Epithelial Cell Proteins by Lysis-Lavage

Fifteen mice were randomly assigned to three treatment groups ($n = 5/\text{group}$) and prepared for airway in situ incubations as described previously by Lin et al. (29) with the modification that the head was removed. Briefly, animals were killed with an overdose of pentobarbital, and the trachea was exposed and cannulated. The entire thorax was removed intact from the animal, and lungs were inflated with 0.5 mL solution consisting of 0.75% w/v Sea Plaque low melting temperature agarose in 5% w/v dextrose, immediately followed by 0.5 mL oxygenated, sulfur amino acid deficient Waymouth's medium containing vehicle (methanol, 10 μ l/ml), 2-methyl-1,4-naphthoquinone (menadione, 200 μ M final concentration), or *t*-butyl hydroperoxide (4 mM final concentration) through a three-way valve. Both solutions were preheated to 37 °C to prevent the agarose from solidifying during inflation. The thorax, containing the inflated lungs, was incubated in Dulbecco's phosphate buffered saline at 4 °C for 10 min to allow the agarose to solidify, and the thorax/lungs were transferred to a beaker containing 37 °C Dulbecco's phosphate buffered saline to incubate for 1 h. Following the incubations, the trachea and lungs were removed from the thoracic cavity, and the Waymouth's media was removed through simultaneous inversion of the lungs and gentle suction with a syringe. Lysis-lavage to recover airway epithelial cell proteins was performed as described previously (28).

All lungs were lavaged with NT-TBL lysis solution containing 1 M NDSB-195, 7 M urea, 4% w/v CHAPS, 0.5% w/v Triton X-100, 25 mM Tris pH 7.6, and 2% v/v PI containing 26.4 mM benzamidine, 100 mM phenanthroline, 0.15 mM aprotinin, 1.46 mM pepstatin, and 2.1 mM leupeptin. Each lavage sample, containing approximately 1 mg protein, was added immediately to tubes containing 250 nmols Cy3 maleimide dye for labeling of reduced thiols within the sample. From this point onward, light exposure was minimized for all CyDye-labeled samples. Cy3 maleimide-labeled samples were incubated at room temperature in the dark overnight (at least 16 h), allowing excess unreacted dye adequate time to hydrolyze within the aqueous lysis solution. Tributylphosphine (40 μ L of 200 mM stock) was added to each sample (24 mM final concentration) and allowed to incubate for 30 min to reduce disulfide bonds and sulfenic acids. Each sample was then reacted with 250 nmols of Cy5 maleimide by incubating at room temperature for at least 2 h.

2DE

Protein concentrations in the maleimide-derivatized samples obtained from lungs incubated in situ were determined according to Bradford (30), with BSA as the standard using the Bio-Rad Protein Assay. Samples were not pooled. IPG buffer was added to 100 μ g of protein from each sample, and sample volumes were brought to 350 μ L with TBL. One narrow range (pH 4.5–5.5) 18 cm Immobiline DryStrip was rehydrated for each sample. Isoelectric focusing of the strips was performed on a Multiphor II at 15 °C at 0.5 mA per strip, gradually increasing the

voltage from 50 to 3500 V over 7 h and running at a constant 3500 V for 23 h (total ~90 kVh). Following focusing, proteins were alkylated with 10 μ M iodoacetamide in SDS equilibration buffer (50 mM Tris-Cl pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS) without further reduction for 30 min prior to electrophoresis in the second dimension. The strips were placed on top of SDS Rhinohide gels (10% T polyacrylamide) 18 cm $\times$ 24 cm $\times$ 1.5 mm and sealed to the gel using 0.5% Isogel agarose containing bromophenol blue. The 2D separation was performed in an Ettan DALTsix electrophoresis unit at 10 $^{\circ}$ C, 8 W/gel for 30 min increased to 17 W/gel until the dye front had migrated 17 to 18 cm. Subsequent to electrophoresis, the protein gels were fixed in 50% v/v methanol, 7% v/v acetic acid overnight.

Protein Visualization and Quantification of Proteins Separated by 2DE

Prior to scanning, gel containers were wrapped in aluminum foil to prevent photobleaching of the fluorescent labels. All 15 protein gels were scanned for Cy3 and Cy5 signal as described above. Each gel was then silver-stained using a method compatible with mass spectrometry to allow comparison of relative amounts of protein on 2 or more gels. All gels were silver-stained as follows: three 20 min washes with deionized H₂O, one 2 min exposure to 0.02% w/v Na₂S₂O₃, two 1 min H₂O washes, 45 min in 0.1% w/v AgNO₃ at 4 $^{\circ}$ C, two 1 min H₂O washes, and a 2 min wash in 2% w/v Na₂CO₃/0.04% v/v HCHO. All solutions were made fresh, and all steps were performed under gentle agitation. Silver-stained gels were scanned on an Epson Expression 1680 flatbed scanner (16 bit grayscale) using the *SilverFast EpsonIT8* software. Gels were scanned at a resolution of 600 dpi. All staining was performed in polymethylpentane 2D gel containers. Gels were warped to align spots using *TT900 S2S* v2006, and spot volume analysis of the gels was performed in *Progenesis PG220* v2006 (both Nonlinear Dynamics). Elementary spot volume calculations were performed in *Microsoft Excel*.

Statistical Analysis

Statistical comparisons of spot volumes between groups were made using a One-Way ANOVA in *SigmaStat 3.0*. Differences were considered significant when $p \leq 0.05$. Six ratios were calculated for each spot: Cy3/Cy5, Cy3/Silver, Cy5/Silver, Cy3/(Cy3+Cy5), Cy5/(Cy3+Cy5), and (Cy3+Cy5)/Silver.

Protein Identification by MALDI-TOF/TOF MS

Proteins identified as being significantly different between treatment groups in at least one of the six calculated ratios were excised from the 2D gels and placed in 96-well ZipPlates (Millipore) containing C18 reverse-phase chromatography media. Gel plugs were destained in the ZipPlate wells using 30 mM K₃Fe(CN)₆ and 100 mM Na₂S₂O₃ mixed in a 1:1 ratio and incubated in the dark for 20 min followed by 3 $\times$ 15 min H₂O washes. Gel plugs were incubated in 25 mM NH₄HCO₃ in 5% acetonitrile for 30 min followed by 2 $\times$ 30 min incubations in 25 mM NH₄HCO₃ in 50% acetonitrile. Gel plugs were incubated in 100% acetonitrile for 10 min before being digested for 4 h at 37 $^{\circ}$ C in 25 mM NH₄HCO₃ containing sequencing-grade, modified trypsin (Promega, Madison, WI) at a final trypsin concentration of 10 μ g/ μ L. The reaction was stopped by the addition of 0.2% trifluoroacetic acid. Samples were eluted in 5 μ L of an aqueous solution containing 65% acetonitrile/2% trifluoroacetic acid using plate centrifugation. Each sample was mixed 1:1 with 8 mg/mL of α -cyano-4-hydroxycinnamic acid in 65% acetonitrile/2% trifluoroacetic acid and spotted to a MALDI target. The MALDI target was analyzed using an Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems Inc., Foster City, CA) with TOF/TOF Optics. MALDI-MS data were collected in the m/z range of 700–4000 using trypsin autolysis peaks of m/z 842.510, 1045.600, 2211.105, and 2239.136 Da as internal calibrants. Data were analyzed using Applied Biosystems *GPS Explorer Software* with a precursor tolerance of 150 ppm, MS/MS fragment tolerance 0.2 Da, variable modifications: oxidation (M), Cy3 (C), Cy5 (C), and a maximum of two missed trypsin

cleavages. The variable modification, menadione (C), was also used as a search criteria for protein spot features that were excised from gels containing a menadione-exposed lung epithelial cell protein sample. The IPI Mouse database searches were performed on all MS and MS/MS data sets using *GPS Explorer Workstation* v3.5 software, and a probability-based Mascot score >60 (equivalent to $p \leq 0.05$) was considered a statistically significant hit.

Results

Stoichiometry of Dye Labeling and Utility in Measuring SH and Disulfide Content of Proteins

Varying concentrations of aldehyde dehydrogenase, trypsinogen, and albumin were reacted with both Cy3 and Cy5 maleimide to determine the stoichiometry of the reaction between the maleimides and model proteins and to demonstrate proportionality between dye labeling and the number of free and disulfide protein thiols. Model protein samples were handled, as described in the methods section, such that native thiols were labeled with Cy3, and internal disulfide bonds as well as sulfenic acids were reduced prior to reaction with Cy5. Model proteins were separated by 1DE. The data in Figure 2 demonstrate a linear response of fluorescent protein band volume versus protein concentration for three different concentrations of aldehyde dehydrogenase and trypsinogen as measured on 1D gels. The fluorescent band volume for thiols and disulfides in BSA was proportional to protein concentration (two concentrations). With all three proteins, the thiol-to-disulfide ratio measured as band intensities for Cy3/Cy5 fluorescence remained constant with increasing protein (part C of Figure 2) and was proportional to the thiol/disulfide ratio for aldehyde dehydrogenase (8 SH, 0 disulfides) and trypsinogen (4 SH and 4 disulfides). Although BSA is reported to have only 1 SH with 17 remaining disulfides, fluorescence analysis of commercial samples indicated 11 or 12 thiols and 12 disulfides (24 thiols after reduction). Further analysis using Ellman's reagent (DTNB) confirmed 12 nanoequivalents of free thiol/nmol protein.

These studies also demonstrated that dye/thiol ratios of less than 2:1 still yielded stoichiometric formation of derivatives. Aldehyde dehydrogenase has 8 free thiols. Even at the highest concentrations of aldehyde dehydrogenase tested (3 nmols protein containing 24 equivalents of thiol reacted with 40 nmols dye) (part A of Figure 2), fluorescence intensity was proportional to protein concentration. The amount of dye needed to react with 1 mg of protein obtained from airway epithelial cells was estimated by reacting samples of rat lung lysis lavage solution with Ellman's reagent. These studies measured approximately 75 nmols SH/mg protein, a value very similar to that reported for protein thiol content of isolated hepatocytes (23). All of the work reported here used 250 nmols dye/sample, thus exceeding the 3:1 ratio of dye to thiol shown to be adequate for complete labeling.

Maleimide Hydrolysis

Initial experiments removed unreacted Cy3 maleimide dye using Biospin columns, but this resulted in considerable dilution of the sample. An alternative approach was to allow the unreacted dye to decompose prior to reduction of the disulfides with TBP and alkylation with Cy5 maleimide. To be certain that the initial Cy3 label was stable in lysis solution, aliquots of protein obtained from rat lung by lysis lavage were reacted with Cy3 and samples were analyzed at 2 h, 16 h, and 16 h + 30 min with TBP solution as described in Methods. Labeled proteins were separated on a 1D gel, and three prominent bands were chosen to determine fluorescence intensity/intensity of silver staining. The results (Figure 3) indicate no significant change ($p \leq 0.05$) in fluorescence band intensity/total protein with time. In addition, the finding that incubation with TBP resulted in no increase in fluorescence supports the fact that the maleimide label has decomposed at 16 h and is no longer thiol-reactive.

Determination of Maleimide Dye-Labeling Bias

The issue of dye bias has been extensively discussed in the microarray literature. This bias appears to be related either to differences in the incorporation of the label in direct labeling methods or to the sensitivity of Cy5 to ozone (31, 32). To determine whether dye bias was an issue in the dual-labeling experiments described here, both maleimides were used to label individual aliquots of a homogeneous rat lysis lavage protein sample. Following protein separation by 1DE, a representative protein band was chosen for use in calculations. The protein band volume was determined for both the Cy3 and Cy5-labeled samples in triplicate. This was normalized to the total amount of protein present in the band determined by silver staining. The average normalized bound fluorescence values differed by 6.1%. This difference was not statistically significant at a p value ≤ 0.05 (Figure 4).

Concentrations of Tributylphosphine Necessary to Yield Optimal Labeling and Disulfide Reduction without Dye Interference

In earlier studies with purified proteins, Tyagarajan et al. (27) showed tris(carboxyethyl) phosphine reducing agent reacted with maleimide and that this reaction could result in decreased intensity of fluorescence in maleimide-labeled protein thiols. Optimal ratios of 1.25:1 for dye to TCEP were reported. The ratios of TBP to dye used in the current studies were considerably greater than those used with individual proteins. Accordingly, three different final concentrations (18.2, 3.92, and 0.995 mM) of tributylphosphine were used to reduce aliquots of a rat lysis lavage protein sample (two of 375 μ g and one of 750 μ g). The best labeling of the protein sample by both Cy3 and Cy5 was determined to be in the sample containing 18.2 mM tributylphosphine when protein band volumes were normalized to the total amount of protein present determined by silver staining (Figure 5).

Alterations in Airway Epithelial Cell Protein in Response to Menadione or *t*-Butyl Hydroperoxide

Menadione and *t*-butyl hydroperoxide are two classic agents used to produce cellular oxidative stress. As further proof of concept that the methods outlined in this manuscript are capable of measuring the oxidation status of protein thiols, in situ incubations of lungs in which airway epithelium was exposed to either menadione or *t*-butyl hydroperoxide were conducted. Following the incubation, airway epithelial cell proteins were removed by lysis lavage. After labeling the complex protein samples with monoreactive maleimide CyDyes, proteins were separated by two-dimensional gel electrophoresis (Figure 6). An average of Cy3 fluorescence/Cy5 fluorescence was calculated for each individual spot feature in each treatment group. Of the average 1912 spot features detected on the gel images 149 were identified that differed significantly ($p \leq 0.05$) in at least one of the six ratios calculated and between at least two of the three treatment groups. Alterations in the Cy3 (reduced)/Cy5 (oxidized) ratio in response to *t*-butyl hydroperoxide or menadione varied significantly ($p \leq 0.05$) among specific spot features interrogated in this study. To help better understand the significance of this value, the ratio change is presented for the Cy3/Cy5 ratio of the protein in the treatment group gels divided by the Cy3/Cy5 ratio of the protein in the control group gels. The dye ratio decreased by as much as 12.5 and 31.7 in airway epithelial cell proteins from lungs incubated with *t*-butyl hydroperoxide and menadione, respectively, compared to control (Figure 7). Not all changes in the dye ratio were negative, and in several cases this ratio increased by as much as 6.3 times control in the *t*-butyl hydroperoxide-treated lungs and 14.9 in the menadione-exposed lungs in comparison to control. Over half of the gel features where Cy3/Cy5 ratios changed most dramatically were not visible with silver staining and therefore could not be reliably selected from the gels nor identified by mass spectrometry. The average ratio change of the Cy3/Cy5 (free/oxidized thiol) ratio for *t*-butyl hydroperoxide was +1.4 and for menadione was -1.2 relative to control. The Cy3/Cy5 ratio was less than control for 35 of the 141 significant spot

features in both treatment groups. Of those 35, 22 had a lower Cy3/Cy5 ratio after menadione treatment and 13 had a lower Cy3/Cy5 ratio after *t*-butyl hydroperoxide treatment. Of the 141 significant protein features, 68 were visible on silver-stained gels (Figure 8) and were excised for identification by MALDI-TOF/TOF MS (Table 1).

All of the proteins identified by MALDI-TOF/TOF MS are listed in Table 1. Although some of the probability-based Mascot scores appear “low”, all scores are above 60, the lowest score possible to identify a protein with a $p \leq 0.05$. Only two databases were used for our protein identification because “different significant levels are obtained if databases of different sizes are searched...” (33). The IPI database balances sequence completeness and degree of sequence redundancy compared with the other most commonly used protein sequence databases (34). The proteins identified by MALDI-TOF/TOF MS are involved in transcription, protein synthesis and folding, and cell death/growth (Figure 9).

Discussion

There is considerable interest in being able to monitor the oxidation status of protein thiols in response to disease and toxicant exposure, and several methods have been presented in the literature. Thorough reviews of currently available methods based on both electrophoresis (35) and mass spectrometry (36) have been published recently. All have advantages and limitations. Several are based on separation of labeled proteins by 2D gel electrophoresis followed by imaging of the radioactive or fluorescent tags. The techniques presented here utilize the DIGE saturation labeling maleimide dyes similar to those presented by Shaw and co-workers (37) in a novel way. These maleimides react with thiols at pH 6.5–7.5 and are closely mass matched, thus allowing oxidized and free thiols to be interrogated from a mixed sample on the same gel and with minimal differential spot migration between treatment groups. Each maleimide molecule contains two sulfonic acid groups. These charged groups cause a shift in the pI of a protein to the more acidic range. 2D gels of pH range 4–7 were run using a subset of the labeled mouse lysis lavage samples described above, and more than 75% of the protein focused within the more acidic range. Therefore, narrow range pH 4.5–5.5 IEF strips were used for the gels run in this study, thus allowing for increased protein loading and less feature overlap on the 2D gels. The primary advantage to the methods presented in this manuscript is that the ratio of both free and oxidized thiols (disulfides, sulfenic acids) can be determined for each sample in a single gel, thereby presenting a global view of changes in the thiol proteome. However, a disadvantage is that the method does not distinguish between a protein where 4 cysteines have been modified by 25% from a protein where a single cysteine residue has been modified so that it no longer reacts with Cy3. These two conditions could result in very different consequences to cellular homeostasis.

Although the concept of multiplexed fluorescence labeling of available thiols followed by reduction of disulfides/sulfenic acids and derivitization of the released thiols appears straightforward, there are several analytical issues that must be addressed to provide confidence that the methods correctly interrogate the thiol/disulfide–sulfenic acid ratios. Thiol/disulfide exchange occurs rapidly and, as pointed out in a recent review (38), has been a major impediment to the correct measurement of soluble thiol/disulfide ratios. The technique used to remove airway epithelial cell proteins results in rapid solubilization of those proteins with strong denaturants. Samples are removed and aliquots are added to maleimide solutions within 1–2 min of the start of lavage. Maleimide dyes are quite reactive at pH 6.5–7.5, and reactions go to completion quickly (27), thus decreasing the chances of thiol/disulfide interchange. Available data suggests that greater than 70% of mammalian proteins contain fewer than 5 cysteines and only 10% have greater than 12 (39). Assuming an average protein molecular weight of 50 kDa, 1 mg of protein would equal approximately 20 nmols. Further assuming that there are 5 free thiols/nmol of protein would mean that there are approximately 100 equivalents

of thiol per milligram of protein. These assumptions are consistent with crude measurements of the thiol content of rat lung lysis lavage samples with DTNB. Experiments showing that fluorescence increases proportionally with increasing protein thiol and disulfide content (after reduction), even where the maleimide/thiol ratio is less than 2:1, support the view that the labeling reagents were present in sufficient excess to provide complete labeling (Figure 2). The calculated maleimide/thiol ratios estimated for our samples, based on reaction of airway epithelial proteins with DTNB, were at least 3:1.

Earlier work with single proteins did not demonstrate an interaction between the maleimide dyes and thiourea (27) even though concerns have been raised (40). Likewise, our work comparing the use of thiourea versus NDSB-195 did not demonstrate any differences in labeling intensity but did result in increased numbers of gel features (by approximately 380). With purified proteins, Tygaragian et al. (27) showed substantial loss of fluorescence if the ratio of reducing agent (TCEP) was too high relative to the amount of dye used, and these authors suggested ratios of dye to reducing agent of 1.25:1. In our work with more complex protein samples, the reducing agent concentrations required for optimal labeling were considerably higher than those observed with purified protein samples (Figure 5).

The goal of developing this method was to identify proteins from a complex sample whose thiol status has changed after treatment by a chemical or chemical mixture. To provide proof of concept for this method, we utilized two classic oxidants, *t*-butyl hydroperoxide and menadione, in systems that we developed for exploring adduction of proteins by reactive metabolites in the lungs. Both of these agents have been shown to produce changes in protein thiol status and, at lower concentrations than those used in the current studies (0.5 mM *t*-butyl hydroperoxide; 0.2 mM menadione), resulted in an increase in glutathione bound as a mixed disulfide to protein (41). Protein glutathione mixed disulfide increased from 0.3 nmols/mg protein in control isolated hepatocytes to 2 and 11 nmols/mg after 30 min incubations with *t*-butyl hydroperoxide and menadione, respectively. In addition, the level of protein glutathionation in incubations with *t*-butyl hydroperoxide was higher at 3 min than at 30 min, whereas this level continues to rise with time in the presence of menadione. These data are consistent with the overall alterations in protein thiol status that we have observed in the current studies.

A spot volume in pixels was calculated for each individual protein feature on each individual gel. Each spot feature had both a Cy3 and Cy5 fluorescence value. These values correspond to the amount of fluorescent maleimide dye that is covalently bound to the thiols on each protein. The Cy3 fluorescent value (in pixels) is proportional to the percentage of thiols on the protein that successfully reacted with the first dye. The Cy5 fluorescent value (in pixels) is proportional to the percentage of oxidized thiols that, upon reduction, successfully reacted with this second dye. Upon successful warping of the gels, the Cy3 and Cy5 fluorescent values for each spot feature were compared across all 15 gels within the experiment. For ease of analysis, the Cy3 and Cy5 values for each spot feature within a treatment group were averaged. The average Cy3/Cy5 ratio of each spot feature in each treatment group was then calculated. These average Cy3/Cy5 fluorescent ratios of spot volumes correspond to the average ratio of free thiols to oxidized thiols of each spot feature in both the control and oxidant-exposed treatment groups that were labeled with fluorescent maleimide dye.

Many of the changes in Cy3/Cy5 ratios in proteins that were detected with silver staining in *t*-butyl hydroperoxide incubations were positive and reflected a decrease in available disulfides compared to the same control protein feature. This positive Cy3/Cy5 ratio change could occur if thiols present as disulfides in the control sample were irreversibly oxidized after incubation with *t*-butyl hydroperoxide. Thiol oxidation to sulfinic and sulfonic acids results in derivatives that cannot be reduced by TBP and thus are unavailable for maleimide labeling. Release of

glutathione from a mixed disulfide bond could also explain some of the dye ratio changes we observed. However, this method was not designed to specifically address the deglutathionation of proteins. Many of the proteins showing a decrease in the Cy3/Cy5 ratio in the *t*-butyl hydroperoxide treatment could not be detected by silver staining, and, thus, these proteins could not be identified. In some cases, this decreased ratio was dramatic, up to 12.5. Because this represents a pool of low-abundance proteins whose thiol oxidation state is altered substantially, we are exploring methods for removing proteins that do not contain cysteine, in an attempt to concentrate samples for detection of these low-abundance, highly oxidized entities.

In contrast, the protein Cy3/Cy5 ratio decreased significantly in the majority of the proteins identified in this study from menadione-treated lung epithelial cells. In only very few cases did the ratio of Cy3/Cy5 labeling increase relative to control. Overall, menadione produced a far more dramatic change in dye ratios than *t*-butyl hydroperoxide. *t*-Butyl hydroperoxide is a direct acting oxidant, and over the 1 h incubation time of this study, proteins could have undergone initial oxidation and recovered or been further oxidized, thus decreasing the Cy5 signal and increasing the apparent Cy3/Cy5 ratio. Menadione undergoes continuous redox cycling, and even though it was present in the incubations in far smaller quantities than *t*-butyl hydroperoxide, it produced a far more dramatic alteration in the dye ratio. Menadione also arylates proteins, (23) and because this likely occurs on protein cysteines, a portion of the available cysteines is removed from Cy3 maleimide labeling.

Although both menadione and *t*-butyl hydroperoxide solutions in Waymouth's medium were made fresh just prior to use, *t*-butyl hydroperoxide is highly reactive and could have partially decomposed prior to being instilled into the lung. Another possibility is that *t*-butyl hydroperoxide targets biomolecules, including lipids and membrane proteins on external cell surfaces, thus resulting in the more moderate changes in those proteins amenable to analysis by the techniques described here. Membrane proteins are difficult to monitor by 2DE and were therefore not likely observed in this study. Finally, menadione is only active within the intracellular space and thus is far more likely to modify proteins that are measured by the methods described here. Of the 141 proteins chosen for further study, only 68 (48.2%) were visible on silver-stained gels. Although silver staining is quite sensitive with a detection limit of about 1 ng, (39) it is often essential to saturate high abundance spots when staining to visualize low-abundance spots because of the silver stain's low dynamic range. Silver nitrate is also one of the more difficult stains to use reproducibly. To minimize this later drawback, every gel in this experiment was stained using the exact same batch of reagents, and all gels were developed in the same amount of time. Because of the high probability of over-staining high abundance proteins, we decided to calculate Cy3 and Cy5 spot volumes relative to silver stain as well as Cy3 and Cy5 spot volumes relative to total fluorescence, minimizing the probability that we would get false-positive significant differences. The identities of the 74 low-abundance spots not seen following silver staining are unknown. The identification of these proteins will require either further concentration of a subset of the proteins or more sensitive detection methods. However, it would be interesting to determine if any of these low-abundance proteins are essential to maintaining the redox homeostasis of the cells.

Many alternative protein stains were tried in an attempt to find a stain that would provide sensitivity similar to silver but with a higher dynamic range: four different published Colloidal Coomassie Blue formulations, Sypro Orange, Sypro Red, Sypro Tangerine, Sypro Ruby, Coomassie R250 with near-IR imaging, and Deep Purple (42). For reasons such as low/no signal or interference of signal with maleimides, none of these total protein stains were compatible with the method outlined here. Although we acknowledge there are limitations with all stains, we concluded that a silver nitrate stain produced the best results in our system given current staining technology.

Of the 60 proteins identified by MALDI-TOF/TOF MS, those involved in transcriptional regulation appeared to be the largest group. A GTP-binding protein was identified as being oxidized by menadione in this study, and a very similar protein was identified as being a target of thiol-reactive electrophiles by Dennehy and colleagues (43). Another primary group of proteins with significant differences in their redox status, as determined by maleimide labeling, were identified as being involved in protein synthesis and folding. This would indicate sensitivity to the redox balance of the cell. This same group of proteins was identified as being a major target of SH-reactive electrophiles (43). A 40S ribosomal protein as well as a eukaryotic translation initiation factor were identified as undergoing S-glutathionylation (44). Our study identified both of these proteins. The later protein we classified as being an inflammatory mediator.

Work from our laboratory has demonstrated that similar proteins are adducted by chemically reactive metabolites generated by P450 metabolism. Wheelock et al. (45) identified specific proteins adducted by 1-nitronaphthalene metabolites in rat lung lysis lavage samples. Of the 14 proteins identified as 1-nitronaphthalene targets, one was identified as serine–arginine-rich SR protein, which is very similar to spot #2779 in our study, the serine–arginine repetitive matrix 2 protein. Other studies, in which naphthalene adducts were identified in mouse liver and lysis lavage samples (46, 30), found ribosomal proteins similar to those shown to be significantly altered in our study. Interestingly, ribosomal proteins were up-regulated in human bronchoalveolar lavage fluid from asthmatics following antigen challenge (47).

Results obtained using the maleimide labeling and multiplexed fluorescence image analysis method described here are consistent with previous work on reactive protein thiols, demonstrating the reliability of this method in investigating complex mixtures. Proteins found to be targeted by known oxidants in this study are those associated with protein synthesis and folding and signal transduction. Whether modification of one or several of these proteins is key to the downstream phenotypes associated with menadione or *t*-butyl hydroperoxide is highly speculative and will require considerable additional effort. Null mice are available for some of the proteins found to be modified, and these may be useful in helping to define a role of these proteins in the toxicity associated with these agents. Further application of this method to tissues exposed to both defined chemicals such as naphthalene and to mixtures may help to discern the roles of oxidative sulfhydryl modification in the toxicity of these agents as well. Toward this end, we are currently utilizing this method to identify proteins differentially oxidized in mouse airway epithelial cells after treatment with naphthalene or diethylmaleate.

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Figure 1. Schematic diagram showing the overall procedure for labeling and measurement of reduced and oxidized protein thiols.

Figure 2.

Linearity of protein labeling when the same amount of Cy3 or Cy5 maleimide dye was added to different concentrations of three purified proteins. The dye/thiol molar ratio was at least 1:1 at concentrations of protein at 4.8, 3.4, and 2.6 μM aldehyde dehydrogenase, 4.8, 3.8, and 2.5 μM trypsinogen, and 1.7 and 0.93 μM BSA. Values reported are from a single labeling reaction where dye-labeled protein samples were separated on a 1D gel.

Figure 3.

Bar graph of a Cy3-labeled protein band after 2, 16, and 16 h, followed by reduction with TBP incubation normalized to the amount of protein present, demonstrating the stability of the fluorescent maleimide derivative. The lack of signal increase following TBP addition shows that no active maleimide remains in the reaction mixture after 16 h. There are no statistical differences between the three treatment groups at $p \leq 0.05$. Values are presented as mean $\pm$ SD, $n = 3$.

Figure 4.

Bar graph of a Cy3 and Cy5-labeled protein band normalized to the amount of protein present showing the equivalency of labeling with these dyes. Fluorescence intensity was evaluated at the same photomultiplier setting for both dyes. There is a 6.1% difference in labeling, which is not statistically significant at $p \leq 0.05$. Values are presented as mean $\pm$ SD, $n = 3$.

Figure 5.

Increased Cy3 and Cy5 labeling with increased tributylphosphine (TBP) concentration. The concentration of TBP used to reduce complex protein samples does not block maleimide labeling. Values are presented as mean $\pm$ SD, $n = 3$.

Figure 6.

Gray-scale images of 2D gels (pH 4.5–5.5) containing proteins isolated from airway epithelium of mouse lungs incubated with vehicle (control) (A), menadione (B), or *t*-butyl hydroperoxide (C). Cy3 images show the relative fluorescent signals of proteins labeled with Cy3 maleimide, and Cy5 images show the relative fluorescent signals of proteins labeled with Cy5 maleimide.

Figure 7.

Variance in positive and negative Cy3/Cy5 ratio changes of individual proteins in treatment groups compared to control, indicating a difference in thiol reactivity to the maleimide Cy dyes within the oxidant-exposed samples. The Cy3/Cy5 ratio change is an indication of a protein's thiol oxidation state at the time of the reaction.

Figure 8. Cy3 scanned image of representative gel with spot map of proteins identified by MALDI-TOF/TOF MS. All 46 proteins are listed in Table 1.

Figure 9.
Pie Chart representing the percentages of identified proteins with known ontogenies.

Table 1

Representative sample of proteins identified by MALDI-TOF/TOF MS. Protein features where chosen for identification when one of the six ratios was significantly different in response to menadione or *l*-butyl hydroperoxide treatment relative to control. Ratio change presented is Cy3/Cy5 ratio of treatment group relative to control

spot #	ratio change w/ <i>l</i> -butyl	ratio change w/ menadione	protein name	TrEMBL Acc. no.	score ^b	peptides ^c	% sequence coverage	no. of Cys ^d	mole % ^e	primary function
1396	+1.13	-1.52	IQ, AAA domain containing protein, isoform 1	Q9CUL5-1 ^f	73	11	31	7	0.817	ATPase activity
1527	+5.36	+1.72	THO complex, subunit 2	IP100357672 ^g	64	25	11	41	2.580	Protein synthesis/folding
1555	+6.31	No data	zinc finger protein 708, isoform C	IP100553389 ^g	64	16	28	40	7.859	transcription
1605 ^a	+1.06	-3.11	zinc finger CCH type containing 13	Q8BHW0	100	26	10	13	0.752	transcription
1632	+2.09	-1.53	GTPase, IMAP family member, isoform A	IP100187462 ^g	84	14	34	6	1.829	cell fate/death
1702	-3.42	-2.41	golgi apparatus protein 1	IP100480573 ^g	68	11	29	4	1.298	cell fate/death
1703	No data	No data	notch 2	Q3TMG0	72	22	20	68	5.787	transcription
1704 ^a	1.00	-3.88	TGF beta inducible nuclear protein 1	A0JNU8	90	12	27	3	1.154	transcription
1726 ^a	+2.90	-1.25	similar to 60S ribosomal protein L29	IP100118362 ^g	68	8	31	2	1.149	protein synthesis/folding
1737	+3.89	-1.10	phosphatidylinositol transfer protein, alpha coiled-coil domain	Q3TGI6	71	11	33	4	1.476	signaling
1805 ^a	+1.07	-3.66	containing protein 77, isoform 1	Q9CZH8-1 ^f	69	16	36	10	2.045	fatty acid metabolism
1890	-1.49	-2.68	similar to ribosomal protein L19	IP100459850 ^g	77	11	40	1	0.476	
1929	-2.67	-2.29	22 kDa protein	IP100623150 ^g	79	7	24			
1969	No data	No data	calmodulin binding transcription activator 1	A2A896	83	8	38	3	1.345	cell fate/death
1970	+1.18	-1.77	68 kDa protein	IP100466314 ^g	75	16	34			
1983	-1.12	+2.32	calmodulin binding transcription activator 1	A2A898	70	8	21	12	2.030	cell fate/death
1994	-1.52	+1.52	hypothetical protein	IP100381932 ^g	74	6	31			
2011	-3.57	-8.44	zinc finger protein 39	Q02525	91	14	61	39	5.432	inflammatory mediator
2016 ^a	+4.37	+1.54	QN1 homologue protein, isoform 1	Q62Q06-1 ^f	78	24	13	7	0.499	cell cycle/growth control
2021 ^a	+2.39	-1.02	similar to ribosomal protein L15	IP100462237 ^g	95	15	35	3	1.250	protein synthesis/folding
2037	-1.25	-2.27	yth domain containing protein 1	Q8C4W4	67	9	15	5	0.679	
2067 ^a	+2.05	-3.28	hypothetical protein	Q2YDW6	65	15	32	44	8.943	transcription
2172	+1.27	-1.68	striatin	IP100352751 ^g	88	20	36	40	8.114	signaling
2183 ^a	+2.11	-5.00	zinc finger protein 3	O08900 ^f	85	11	25	1	0.625	transcription
2184 ^a	+1.76	-3.44	zinc finger protein 760	Q5U4A6	78	19	31	54	8.108	transcription
2195	No data	No data	monoglyceride lipase	Q9D976	79	13	37	6	2.521	fatty acid metabolism

spot #	ratio change w/ butyl	ratio change w/ menadione	protein name	TrEMBL Acc. no.	score ^b	peptides ^c	% sequence coverage	no. of Cys ^d	mole % ^e	primary function
2213	-1.25	-2.00	zinc finger protein 758	Q6PHA7	102	23	50	52	8.919	transcription
2237 ^a	+1.97	+1.69	calmodulin binding protein Shal	Q8CJ27	104	42	9	70	2.242	cell cycle/growth control
2271	-2.98	-2.84	similar to 60S ribosomal protein L29	IP100277330 ^g	74	8	42	1	0.625	protein synthesis/folding
2292	No data	-6.92	similar to 60S ribosomal protein	IP100458061 ^g	74	11	25	4	1.465	protein synthesis/folding
2310	No data	No data	similar to pORF2	O08906	61	16	15	18	1.405	DNA polymerase activity
2377 ^a	No data	-2.14	ankyrin repeat domain containing protein 11	Q3UMF4	85	26	7	37	1.389	transcription
2393 ^a	-3.63	-2.17	coiled-coil domain containing protein 34	Q3UI66	69	11	18	7	1.907	
2475	-8.38	-31.67	hypothetical protein	Q6NZP4	69	15	27	42	8.284	
2539	-1.27	No data	hypothetical protein	Q9D4J2	75	10	39	8	4.020	
2567 ^a	+3.37	-2.09	eukaryotic translation initiation factor 3, subunit 10	Q3TKF9	75	25	16	7	0.521	inflammatory mediator
2574	+5.81	+1.67	Sjogren syndrome antigen B	A2AV01	93	15	29	3	0.787	transcription
2615	No data	+14.91	probable global transcription activator	A2AEF5	67	20	16	12	1.130	cell cycle/growth control
2779	No data	-4.09	SNF2L1, isoform 2	Q3TR34	73	10	13	1	0.203	protein synthesis/folding
2813	+1.28	No data	serine/arginine repetitive matrix 2	Q8CEM3	89	18	13	20	1.957	Signaling
2886 ^a	No data	-2.72	Polyamine modulated factor 1 binding protein 1	A2A547	73	9	31	2	1.031	protein synthesis/folding
2916	+1.06	-2.60	ribosomal protein L19	Q3TPJ4	76	13	12	7	1.104	transcription
2930	-6.25	-17.99	nucleolar GTP-binding protein 1	IP100605760 ^g	63	8	39			protein synthesis/folding
2934	No data	-19.08	similar to 40S ribosomal protein S6	A0JLM6	121	29	16	11	0.892	cell fate/death
2983 ^a	No data	No data	structural maintenance of chromosome 1A	Q3U365	75	14	15	19	3.381	transcription
3000	No data	+1.19	zinc finger protein 512	IP100468954 ^g	63	9	40			

^aThe Cy3/Cy5 ratio is statistically significantly different between at least two of the three treatment groups.

^bThe probability-based Mascot score.

^cThe number of peptides used for database matching.

^dThe number of cysteines present on the protein according to the International Protein Index.

^eThe molar percentage of the cysteines in the protein according to the International Protein Index.

^fA UniProtKB/Swiss-Prot accession number.