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ARTICLE *in* BIOCHEMISTRY · OCTOBER 2000

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Identification of Oxidant-Sensitive Proteins: TNF-α Induces Protein Glutathiolation

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Received April 4, 2000; Revised Manuscript Received June 23, 2000

ABSTRACT: Reactive oxygen species are thought to play a role in a variety of physiologic and pathophysiological processes. One possible mediator of oxidant effects at the molecular level is a subset of proteins containing reactive cysteine thiols that can be readily oxidized. The transient incorporation of glutathione into cellular proteins is an established response to oxidant stress and could provide a mechanism for reversible covalent modification in response to reactive oxygen species. To better understand the function of protein S-glutathiolation in vivo, a biotinylated membrane-permeant analogue of glutathione, biotinylated glutathione ethyl ester, was developed and used to detect proteins into which glutathione is incorporated under oxidant stress. Oxidant stress from exogenous hydrogen peroxide or generated in response to TNF-α was found to increase incorporation of biotinylated glutathione ethyl ester into several HeLa cell proteins. The identity of two of these proteins was determined by peptide sequencing and mass spectrometric peptide mapping. A 23 kDa S-glutathiolated protein was identified as thioredoxin peroxidase II, a member of the peroxiredoxin family of peroxidases known to play a role in redox-dependent growth factor and cytokine signal transduction. A second, 36 kDa, protein was identified as annexin II. Further investigation revealed a single reactive cysteine in the annexin II tail domain. Deletion of the identified cysteine was found to abolish S-glutathiolation of annexin II. These findings demonstrate a specific posttranslational modification associated with an endogenously generated oxidant stress and suggest a mechanism by which TNF-α might selectively regulate protein function in a redox-dependent fashion.

There is now a considerable body of evidence supporting the notion that reactive oxygen species (ROS)[1] mediate cellular responses to many important physiological stimuli. A variety of agonists including peptide growth factors and cytokines (*1*−*5*) and direct activators of signal transduction pathways such as constitutively active mutants of Ras and Rac and phorbol ester (*6*−*8*) have been shown to induce production of ROS. The ROS produced have, in turn, been identified as requisite mediators of downstream signaling events in these pathways (*4*, *5*, *8*). Despite strong evidence implicating ROS in signaling processes, the precise role of oxidants, such as superoxide and hydrogen peroxide (H_2O_2), in signal transduction is still a matter of considerable controversy. Much of the uncertainty stems from a scarcity of information related to how redox regulation is accomplished at the molecular level. The exquisitely controlled nature of ROS-mediated responses, such as proliferation and apoptosis, suggests the existence of a mechanism by which ROS can induce reproducible changes in the functioning of a select group of signaling-related molecules. While the existence of such a mechanism might have previously been thought unlikely due to the highly reactive nature of oxygen radicals, recent studies have begun to demonstrate how redox signaling could be accomplished. Examples include the reversible activation of the bacterial transcription factor OxyR through oxidant-dependent formation of an intramolecular disulfide bond (*9*) and the reversible inactivation of protein−tyrosine phosphatase 1B through oxidation of a cysteine in its active site (*10*, *11*). In both cases oxidation−reduction of protein cysteinyl thiols provides the on−off switch.

In the case of PTP-1B, the oxidized cysteine is stabilized through the formation of a mixed disulfide with glutathione. Formation of the mixed disulfide prevents the irreversible oxidation of the thiol to a sulfinic or sulfonic acid and allows for the reactivation of the enzyme by cellular thioreductases. This type of reversible S-glutathiolation is not unique to PTP-1B. S-Glutathiolation of a discrete number of proteins is an established response to oxidant stress and has been shown to alter the function of a number of proteins in vitro (reviewed in ref *12*). Unfortunately, progress in understanding the physiological significance of S-glutathiolation has been slow. In most cases the evidence supporting S-glutathiolation as a regulatory mechanism is indirect, and progress has been hindered by the inability to directly identify proteins that are S-glutathiolated in vivo (*12*).

In this study we demonstrate that a membrane-permeant analogue of glutathione, biotinylated glutathione ethyl ester (BioGEE), can be used as an effective marker for oxidant-induced S-glutathiolation. Using BioGEE, protein S-glutathiolation is shown to accompany TNF-α stimulation in

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[1] Abbreviations: BioGEE, biotinylated glutathione ethyl ester; DMEM, Dulbecco's modified Eagle medium; DHR123, dihydrorhodamine 123; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IAM, iodoacetamide; PAGE, polyacrylamide gel electrophoresis; ROS, reactive oxygen species; TPxII, thioredoxin peroxidase II.

10.1021/bi0007674
Published on Web 08/16/2000

HeLa cells, and two of the proteins labeled with BioGEE in association with TNF-α stimulation are identified as annexin II and thioredoxin peroxidase II (TPxII). Both of the identified proteins have been implicated as modulators in signal transduction pathways. These findings both demonstrated the effectiveness of the technology described here and raise the possibility that protein S-glutathiolation is a physiological component of TNF-α signaling.

EXPERIMENTAL PROCEDURES

Materials. EZ-link sulfo-NHS-biotin, D-biotin, Ellman's reagent, BCA protein assay reagent, and GelCode blue were purchased from Pierce (Rockford, IL). Glutathione ethyl ester, *N*-ethylmaleimide, cycloheximide, iodoacetamide, and streptavidin immobilized on 4% beaded agarose were purchased from Sigma (St. Louis, MO). The SilverXpress silver staining kit and polyacrylamide gels were purchased from Novex (San Diego, CA), L-[^{35}S]cysteine (>1000 Ci/mmol) was from Amersham Pharmacia Biotech (Piscataway, NJ), and ebselen was obtained from Calbiochem (La Jolla, CA). Dulbecco's modified Eagle medium (DMEM), containing high glucose, L-glutamine, pyruvate, and pyridoxine hydrochloride, and Hank's balanced salt solution were purchased from Life Technologies (Gaithersburg, MD), and lysyl endopeptidase was from Wako Chemicals (Richmond, VA). Enlightning was purchased from DuPont-NEN (Boston, MA). Monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase antibody was obtained from Advanced ImmunoChemical, Inc. (Long Beach, CA), monoclonal anti-Bcl-2 (100) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), and rabbit anti-TPxII antiserum was generously provided by Dr. Sue Goo Rhee (NIH).

Cell Culture and Transfections. HeLa cells were grown in DMEM containing 10% fetal bovine serum supplemented with penicillin and streptomycin. For creation of a stable Bcl-2-expressing line, cells were transfected with a wild-type Bcl-2 construct (described previously ref *13*) using lipofectamine according to the manufacturer's protocol. Clonal lines were established by limiting dilution and selection with 500 μg/mL G418 and screened for Bcl-2 expression by western blot.

Wild-type and C9G annexin II cDNAs (generously provided by Kathryn Hajjar, Cornell University Medical College) were subcloned into the plasmid pcDNA4-myc/his (InVitrogen, Carlsbad, CA) to produce a C-terminal fusion with the myc epitope tag. The tagged proteins were expressed in HeLa cells by transient transfection using lipofectamine and the manufacturer's protocol.

Labeling of S-Glutathiolated Proteins with L-*[^{35}S]Cysteine*. Oxidant-stimulated incorporation of low molecular weight thiols into soluble protein was assayed essentially as described (*14*). HeLa cells in confluent 6-cm dishes were starved overnight in serum-free DMEM, and the following day the medium was changed to Hank's balanced salt solution containing 250 μg/mL cycloheximide. Following a 1 h incubation with cycloheximide at 37 °C, 25 μCi of L-[^{35}S]-cysteine was added to the medium, and the incubation was continued for an additional 1 h. The labeled cells were then exposed to H_2O_2 at the indicated concentration for 10 min. Incubations were terminated by removing the medium and washing twice with ice-cold PBS. A buffer containing 1 mM EDTA, 1 mM EGTA, 50 mM *N*-ethylmaleimide, 40 μg/mL aprotinin, 40 μg/mL leupeptin, and 50 mM Tris-HCl, pH 7.0, was added immediately after washing, and cells were harvested into microcentrifuge tubes by scraping. Cells were lysed by freezing in a dry ice−ethanol bath and thawing, and insoluble material was removed by centrifugation for 15 min at 14000*g* and 4 °C. Protein concentration in the supernatant was determined using BCA protein assay reagent, and 40 μg of protein was resolved by SDS−polyacrylamide gel electrophoresis (PAGE) in 4−20% gradient gels under nonreducing conditions or after a 30 min incubation with 10 mM DTT. The gels were fixed with water/methanol/acetic acid (45:45:10) and enhanced with Enlightning before being dried for autoradiography.

Biotinylation of Glutathione Ethyl Ester. Sulfo-NHS-biotin, a biotinylating reagent with selectivity for primary amines, was reacted with glutathione ethyl ester at a 1:1 molar ratio in 50 mM $NaHCO_3$, pH 8.5. After 1 h at room temperature the reaction was terminated, and any remaining biotinylation reagent was quenched by the addition of NH_4HCO_3 to a 5-fold molar excess of the starting sulfo-NHS-biotin concentration. Mass spectrometric analysis of the crude reaction mixture confirmed the addition of biotin to the γ-glutamyl residue of glutathione ethyl ester. The mixture was also found to contain unreacted glutathione ethyl ester and free biotin. When NHS-biotin was reacted with glutathione ethyl ester at greater than a 1:1 molar ratio, a significant amount of glutathione ethyl ester was found to have a second molecule of biotin incorporated at the −SH group via a thioester bond. Therefore, to confirm that this dibiotinylated species was not a significant product of the biotinylation reaction, the concentration of reduced glutathione in the reaction mixture was quantified using Ellman's reagent. When glutathione ethyl ester was reacted with NHS-biotin at less than or equal to 1:1 (mol/mol), the concentration of free −SH in the reaction mixture did not change over the course of the reaction. For intact cell assays, the reaction mixture was added to the medium at a final concentration of approximately 250 μM free −SH (a 1:100 dilution of the reaction mixture).

Purification of Putative S-Glutathiolated Proteins Using BioGEE. Confluent 10 cm dishes were starved overnight in serum-free DMEM, and immediately prior to the addition of BioGEE, the medium was again replaced with fresh DMEM. BioGEE was added to the medium 1 h prior to the addition of H_2O_2 at the concentrations indicated in the figures or concurrent with the addition of TNF-α (10 ng/mL) with or without cycloheximide (1 μg/mL). Incubations were terminated, and a soluble protein extract was obtained exactly as described above for the [^{35}S]cysteine assay.

Proteins covalently bound to biotin were extracted in batch using the following procedure: First, soluble protein was clarified by incubation for 30 min at 4 °C with streptavidin−agarose (50 μL/mg of soluble protein) that had been blocked by incubation with D-biotin in 100-fold excess of the biotin binding capacity of the streptavidin−agarose beads. The clarified protein was then incubated with streptavidin−agarose (100 μL/mg of protein) for 30 min at 4 °C. The agarose beads were washed 4 times with RIPA buffer (1% NP-40, 0.1% SDS, 0.5 mg/mL sodium deoxycholate, 150 mM NaCl, and 50 mM Tris-HCl, pH 7.5) and twice with PBS containing 2 mM EDTA and 0.1% SDS. Proteins bound

to streptavidin via a disulfide bond were then eluted from the beads by incubation for 30 min with PBS/EDTA/SDS containing 10 mM DTT. Proteins in the eluent were resolved by SDS−PAGE and detected by western blotting or by staining with silver (SilverXpress) or Coomassie (GelCode blue) according to manufacturers' protocols.

Dihydrorhodamine 123 (DHR123) Fluorescence. Control cells or cells stimulated for 4 h with TNF-α (10 ng/mL) and cycloheximide (1 μg/mL) were washed with Hank's BSS and then incubated for 15 min at 37 °C in Hank's BSS containing 5 μg/mL DHR123. Following the incubation with DHR123, the cells were washed with Hank's BSS and imaged using a Nikon TE300 inverted microscope and IP Lab version 3.2.4 software (Scanalytics, Inc., Fairfax, VA). Fluorescence was quantified from 50 to 100 random cells obtained from three separate fields and expressed as the relative mean fluorescence compared to control cells.

Protein Identification. Soluble protein was obtained from cells that had been incubated in the presence of BioGEE, TNF-α (10 ng/mL), and cycloheximide (1 μg/mL) as described above. A streptavidin extract prepared from 5 mg of soluble protein was resolved by SDS−PAGE on a 12% acrylamide gel. Proteins were detected using Coomassie stain, and bands were excised from the gel. In-gel digestions with lysyl endopeptidase (Lys-C) were carried out using a modification of the method published by the Association for Biomedical Resource Facilities at http://www.abrf.org/ABRF/ResearchCommittees/intprotseqrescom.html. Excised bands in 1.5 mL Eppendorf-type tubes were destained by incubation overnight with 1 mL of 50% methanol and 10% acetic acid. The gel slices were washed with 1 mL of 30% methanol for 1 h and then with 500 μL of 100 mM NH_4HCO_3 for 1 h. Cysteines were reduced by incubation with 3 mM DTT in 100 mM NH_4HCO_3 for 30 min at 60 °C. After the solution was cooled to room temperature, 5.9 mM iodoacetamide was added, and samples were held in the dark for 30 min. Gel slices were washed with 1:1 acetonitrile/0.1 M Tris, pH 9.2, for 1 h and then shrunk in 50 μL of acetonitrile and dried briefly. Ten microliters of 0.1 M Tris, pH 9.2, containing 0.1 μg of Lys-C was added, and following absorption of the digestion buffer into the gel, 20 μL of buffer without enzyme was added. The gel slices were incubated for 18 h at 37 °C with gentle rocking and centrifuged, and the supernatants were transferred to a glass autosampler vial. The gel slices were extracted twice with 150 μL of 0.1% trifluoroacetic acid and 60% acetonitrile for 1 h at room temperature, and the liquid phases were added to the autosampler vial. The extract was dried in a vacuum centrifuge and redissolved in 50 μL of 0.05% trifluoroacetic acid and 5% acetonitrile.

The peptide mixture was separated by reverse-phase HPLC with both spectrophotometric and mass spectrometric detection (Hewlett-Packard Model 1100; Palo Alto, CA) using a Vydac narrow bore C18 column (no. 218TP5205, Vydac; Hesperia, CA). The initial solvent was 0.05% trifluoroacetic acid and 5% acetonitrile with elution by acetonitrile/0.05% trifluoroacetic acid at 1%/min and a flow rate of 0.2 mL/min. Peptide sequences were determined by automated Edman degradation with a Hewlett-Packard G1005 sequencer running version 3.5 of the manufacturer's chemistry program. For peptide mapping by mass spectrometry, the effluent from the spectrophotometric detector was mixed in a tee with 100 μL/min acetic acid pumped by another model 1100 pump, and the mixture was introduced into the mass spectrometer (*15*). The capillary voltage was 4500 V, and the fragmentor was programmed to ramp from 50 V at 50 nm, to 80 V at 1500 nm, and to 140 V at 2500 nm. Data were collected from 550 to 2000 nm.

Iodoacetamide (IAM) Labeling of Annexin II. Thirty micrograms of lyophilized annexin, 36 kDa, a commercial extract from bovine lung containing predominantly annexin II, was dissolved in reaction buffer [140 mM NaCl, 4 mM KCl, and 50 mM sodium phosphate (pH 6.0 at 37 °C)] and dialyzed against reaction buffer to remove DTT present in the commercial preparation. The dialyzed protein was split into two aliquots and warmed to 37 °C. IAM was added to one aliquot to give a final concentration of 20 μM, and the reaction was allowed to proceed for 10 min at 37 °C. The reaction was terminated with the addition of DTT and ETDA to final concentrations of 200 μM and 1 mM, respectively. The protein solution was adjusted to pH 8.5 with 1 M NaOH and transferred to a glass autosampler vial for reduction and alkylation, which was performed with hexafluoro-2-propanol rather than guanidine or urea to increase recovery of the annexin II.[2] The solution was adjusted to 0.1 M Tris, 1 mM EDTA, pH 8.5, and 10 mM DTT. An equal volume of hexafluoro-2-propanol was added, giving a final DTT concentration of 5 mM, and the samples were incubated at 37 °C for 30 min. Acrylamide was added to a concentration of 20 mM, and the incubation was continued for an additional 30 min. An additional 10 mM DTT was added, and the samples were incubated another 5 min. The samples were dried in a vacuum centrifuge, taken up with 50 μL of water, and digested with 0.1 μg of Lys-C overnight at 37 °C. The peptide mixture was again reduced and alkylated as above to ensure full alkylation of cysteine residues. The samples were then dried and redissolved in 50 μL of 10% acetonitrile. The peptides were mapped on the HPLC−mass spectrometer as described for the in-gel digestion. The cysteine-containing peptides were identified by their extracted ion chromatographs for both the iodoacetamide and acrylamide derivative masses. The mass/charge ratio was 1 for P329-338, 2 for P1-9, and 3 for P119-147, selected to give a ratio between 550 and 1200. For the propionamide and carboxymethylamido derivatives the values were 1111.2/1097.2, 572.2/565.1, and 1137.3/1132.6, respectively, for the three peptides.

RESULTS

Procedure for in Situ Labeling of Soluble Proteins with BioGEE. The reagent BioGEE was developed on the basis of previous studies indicating plasma membrane permeability of the L-γ-glutamyl-L-cysteinylglycyl ethyl ester derivative of glutathione (*16*) and the availability of simple and reliable biotin−streptavidin solid-phase extraction techniques. Using this reagent, the scheme diagrammed in Figure 1A was devised to obtain partial purification of proteins into which BioGEE has been incorporated. Consistent with labeling of intracellular proteins prior to plasma membrane lysis, it was necessary to preincubate cells with BioGEE for incorporation into soluble protein since no incorporation was detected when the reagent was added immediately before the cells were lysed (data not shown). When cells were incubated with BioGEE for 1 h, it was possible to extract a distinct set of

[2] Unpublished observation.

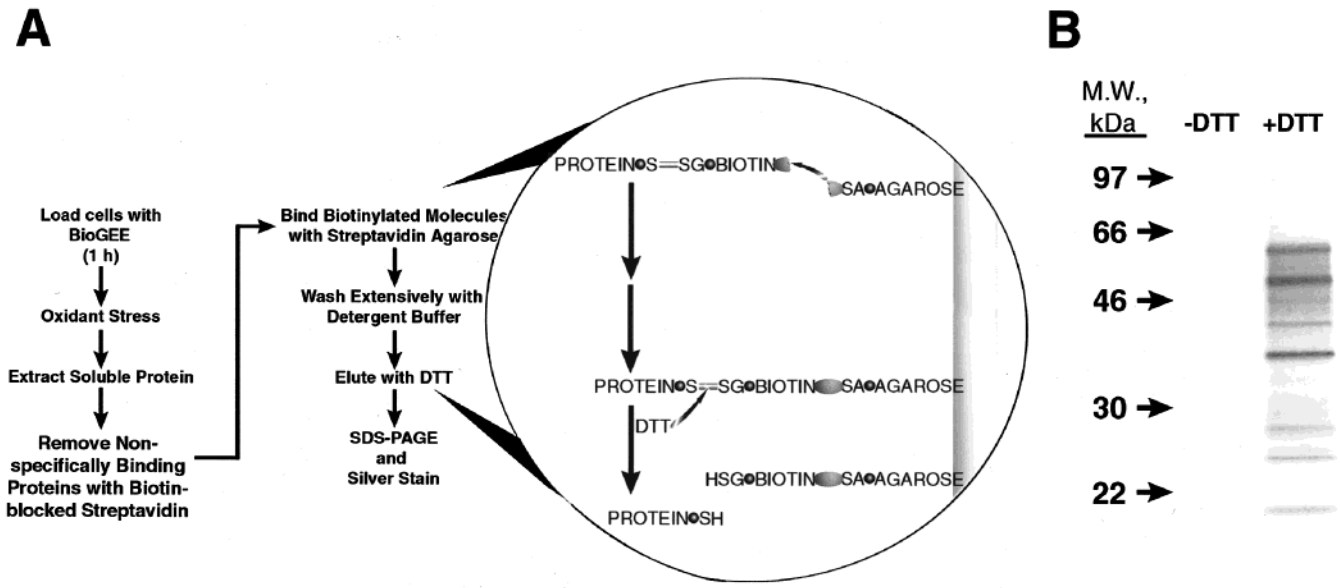

FIGURE 1: Panel A: Diagram of the protocol devised for labeling and purification of S-glutathiolated proteins with BioGEE. Abbreviations: S, sulfur; SG, glutathione; SA, streptavidin; DTT, dithiothreitol. Panel B: Purification of putative S-glutathiolated proteins. A soluble protein extract was obtained as outlined above and in Experimental Procedures from HeLa cells incubated in the presence of H_2O_2 (10 mM, 10 min). Biotin-containing proteins were extracted from 0.5 mg of soluble protein with streptavidin−agarose. The beads were washed and eluted sequentially first with PBS containing 0.1% SDS (−DTT eluate) and then with PBS containing 0.1% SDS and 10 mM DTT (+DTT eluate). The eluates were resolved by SDS−PAGE, and proteins were detected by silver staining of the gel.

proteins from a soluble protein lysate using streptavidin−agarose and elution with DTT (Figure 1B). These proteins did not elute when DTT was omitted from the elution buffer, indicating that biotin has been incorporated into protein through a disulfide linkage with the glutathione moiety of BioGEE.

H_2O_2 Dependence of BioGEE Incorporation. Both the number of proteins and the amount of individual proteins eluting from the streptavidin−agarose beads were found to increase when cells were exposed to H_2O_2, and this increase was found to be dependent upon the concentration of H_2O_2 used (Figure 2A). The oxidant dependence of incorporation suggests that BioGEE is labeling proteins that are S-glutathiolated under oxidant stress. To support this assertion, the results obtained with BioGEE were compared to results obtained using L-[^{35}S]cysteine in the presence of cycloheximide, a classical marker for protein S-glutathiolation. When HeLa cells were loaded with L-[^{35}S]cysteine under conditions that promote its incorporation specifically into low molecular weight thiols, H_2O_2 was found to induce ^{35}S labeling of soluble proteins with molecular weights that are similar to the more prominent bands detected using BioGEE (Figure 2B). In addition, the H_2O_2 concentration dependence of L-[^{35}S]cysteine incorporation into individual proteins was found to be similar to that of BioGEE. For example, there is little or no measurable incorporation of either label into the two proteins that run less than 23 kDa at H_2O_2 concentrations below 10 mM, while incorporation of both BioGEE and L-[^{35}S]cysteine into several other proteins increases in a more or less linear fashion over the entire H_2O_2 concentration range. The similarity in oxidant concentration dependence is consistent with incorporation of both labels into protein via the same mechanism.

Oxidant-Dependent Incorporation of BioGEE into Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH). To verify that BioGEE could detect relevant S-glutathiolated proteins,

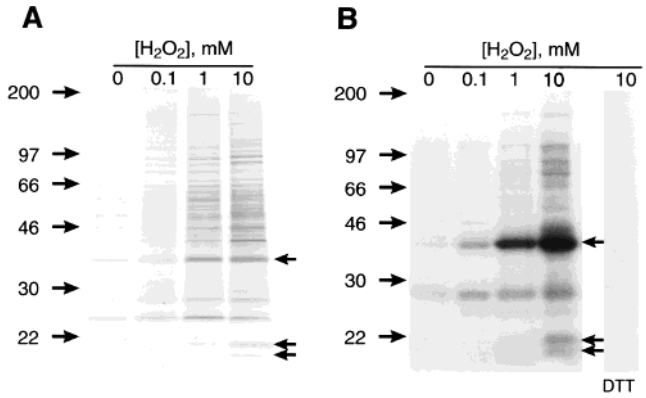

FIGURE 2: Oxidant dependence of BioGEE and L-[^{35}S]cysteine incorporation into soluble protein. Panel A: HeLa cells were loaded with BioGEE and subsequently exposed to H_2O_2 at the indicated concentrations for 10 min. Biotin-containing proteins were extracted from 0.5 mg of soluble protein as described in Figure 1 and Experimental Procedures. Proteins eluting with DTT were resolved by SDS−PAGE and detected by silver staining of the gel. Panel B: HeLa cells were loaded with L-[^{35}S]cysteine in the presence of cycloheximide and subsequently exposed to H_2O_2 at the indicated concentrations for 10 min. Forty micrograms of soluble protein was resolved by SDS−PAGE under nonreducing conditions, and ^{35}S incorporation into protein was detected by autoradiography. Forty micrograms of protein from the 10 mM H_2O_2 sample was also run under reducing conditions to confirm incorporation of L-[^{35}S]-cysteine via disulfide linkage (DTT). Arrows to the right of each panel indicate bands specifically referred to in the text.

the streptavidin extract was transferred to nitrocellulose, and the membrane was probed with an antibody against GAPDH, a protein for which there is a considerable amount of evidence to support oxidant-dependent S-glutathiolation (*12*, *14*, *17*). The amount of GAPDH detected in the streptavidin extract was found to increase with increasing H_2O_2 concentration, and addition of the glutathione peroxidase mimetic ebselen was found to prevent the incorporation of BioGEE into GAPDH (Figure 3). These data are consistent with

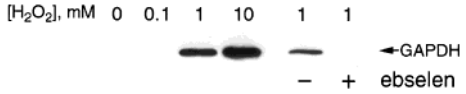

FIGURE 3: Oxidant-dependent incorporation of BioGEE into glyceraldehyde-3-phosphate dehydrogenase. HeLa cells were loaded with BioGEE in the presence or absence of 25 μM ebselen and exposed to H_2O_2 at the indicated concentrations for 10 min. Streptavidin extracts from 1 mg of protein were resolved by SDS−PAGE and transferred to nitrocellulose for western blotting with a monoclonal antibody against GAPDH.

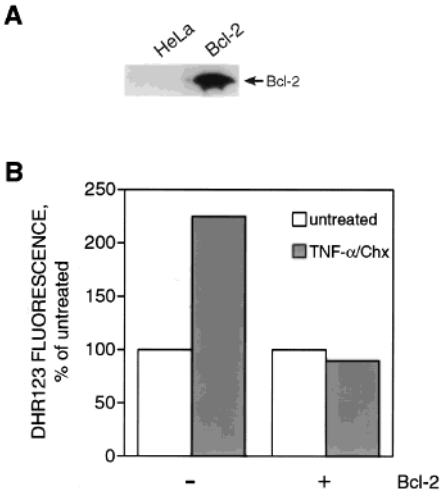

FIGURE 4: TNF-α-induced mitochondrial oxidant production in wild-type and Bcl-2 overexpressing HeLa cells. Panel A: Ten micrograms of soluble protein obtained from parental HeLa cells or a clonal line stabily transfected with a Bcl-2 cDNA was probed with an antibody against Bcl-2. Panel B: Cell-associated DHR123 fluorescence was quantified, as described in Experimental Procedures, in parental and Bcl-2-expressing HeLa cells that were either untreated or stimulated with 10 ng/mL TNF-α and 1 μg/mL cycloheximide for 4 h.

previous descriptions of glutathione incorporation into GAPDH under oxidant stress and strongly suggest that incorporation of BioGEE can be used as a marker for S-glutathiolated proteins.

TNF-α-Stimulated S-Glutathiolation and Identification of Annexin II and TPxII. In an attempt to identify proteins that might be regulated by S-glutathiolation in conjunction with a physiological redox stress, incorporation of BioGEE was measured in cells undergoing apoptosis in response to treatment with TNF-α, a redox-dependent process in HeLa cells (*18*). The TNF-α-induced rise in mitochondrial oxidant production was confirmed using the redox-sensitive dye DHR123 (Figure 4B). Consistent with its relation to proapoptotic TNF-α signaling, the rise in mitochondrial oxidants was blocked by overexpression of the antiapoptotic protein Bcl-2 (Figure 4). When BioGEE incorporation into protein was analyzed in TNF-α-stimulated HeLa cells, several of the bands detected in the streptavidin extract were found to increased significantly compared to cells incubated with BioGEE alone (Figure 5). This increase in BioGEE incorporation was also blocked by overexpression of Bcl-2.

The assay was then scaled up to obtain a sufficient quantity of protein for identification of putative S-glutathiolated proteins. A streptavidin extract was obtained from 5 mg of soluble protein from TNF-α-stimulated cells, and proteins that could be visualized by Coomassie staining were excised for in-gel digestion with Lys-C. Peptides obtained from a

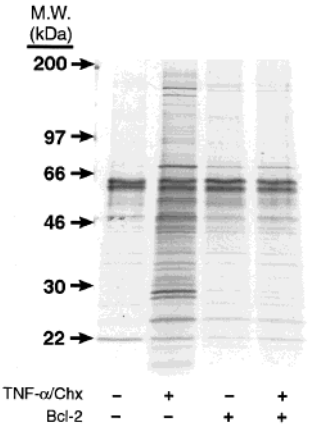

FIGURE 5: TNF-α-induced incorporation of BioGEE into soluble protein. Soluble protein was obtained from parental or Bcl-2-expressing HeLa cells incubated for 4 h with BioGEE alone or BioGEE in the presence of 10 ng/mL TNF-α and 1 μg/mL cycloheximide. Biotin-containing proteins were extracted from 0.5 mg of soluble protein as described in Figure 1 and Experimental Procedures. Proteins eluting with DTT were resolved by SDS−PAGE and detected by silver staining of the gel.

23 kDa band were separated by reverse-phase HPLC, and Edman sequencing was performed on two peaks eluting at approximately 15% and 17% acetonitrile. Both peptides were sequenced through to the C-terminal lysine and unambiguously identified the protein as TPxII (P28-35, DISLSDYK; P110-120, RTIAQDYGVLK). The molecular mass of TPxII, 22 110 Da, is in good agreement with that estimated from the SDS gel.

The entire Lys-C digest of an approximately 38 kDa band was analyzed by HPLC−mass spectrometry since the amount of protein was relatively limited. Four peaks were clearly detected, which were not present in a blank digest, with masses of 1421.1, 2154.9, 3140.1, and 3577.5. These were searched against the National Center for Biomedical Research databank using the ProFound program (http://prowl.rockefeller.edu/), yielding identification of the protein as human annexin II with probability >99%. The molecular mass of human annexin II is 38 473 Da, again in reasonable agreement with that estimated from the SDS gel.

Western blots of soluble protein−streptavidin extracts confirmed the redox-dependent incorporation of BioGEE into annexin II and TPxII (Figure 6). Treatment of HeLa cells with TNF-α in the presence of cycloheximide resulted in a significant increase in the amount of each protein detected in the streptavidin extract as compared with extract obtained from unstimulated cells. Interestingly, a smaller, but still significant, increase in TPxII S-glutathiolation was also detected when cells were stimulated with TNF-α in the absence of cycloheximide, in which case the cells do not undergo apoptosis. This finding supports the notion of a lower level oxidant burst associated with nonlethal TNF-α signaling. Consistent with redox-dependent S-glutathiolation, H_2O_2 was also found to increase the amount of both TPxII and annexin II in the streptavidin extract in a concentration-dependent fashion (Figure 6).

Identification of a Reactive Cysteine in Annexin II. The identification of annexin II as a target for S-glutathiolation implies that the protein contains at least one reactive cysteine. To identify this residue, purified bovine annexin II was exposed to a low concentration (20 μM) of iodoacetamide

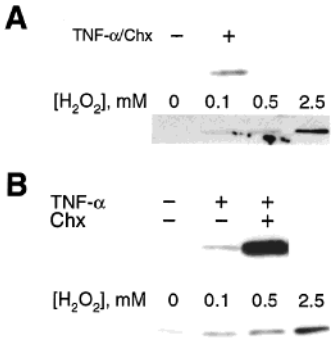

FIGURE 6: TNF-α- and H_2O_2-induced incorporation of BioGEE into thioredoxin peroxidase II and annexin II. Soluble protein was obtained from HeLa cells incubated with BioGEE alone or BioGEE in the presence of the indicated stimuli (10 ng/mL TNF-α plus or minus 1 μg/mL Chx, 4 h; H_2O_2 at the indicated concentrations, 15 min). Biotin-containing proteins were extracted from 0.5 mg of soluble protein as described in Figure 1 and Experimental Procedures. Proteins eluting with DTT were resolved by SDS−PAGE and transferred to nitrocellulose for western blotting with antibodies against annexin II (panel A) or TPxII (panel B).

at pH 6.0. Under these conditions, only a "reactive" cysteine will be labeled, that is, a cysteine whose local environment allows ionization to the thiolate at this low pH. Of the cysteine-containing peptide fragments recovered following Lys-C digestion, only Cys-9 had reacted with iodoacetamide (Figure 7A). This finding suggests that Cys-9 is the most likely target of S-glutathiolation in the annexin II molecule. To confirm this, a mutant of annexin II in which Cys-9 is replaced with glycine was tested for reactivity with BioGEE in vivo (Figure 7B). To distinguish the mutant protein from endogenous annexin II, cDNAs encoding mutant and wild-type annexin II were subcloned in-frame with a myc epitope tag, and streptavidin extracts were probed with an anti-myc antibody. Transient transfection of the annexin II−myc cDNAs into HeLa cells resulted in approximately the same level of protein expression from both the mutant and wild-type constructs. Consistent with the results obtained with iodoacetamide, only the wild-type protein was labeled with BioGEE, indicating that Cys-9 is the site of incorporation.

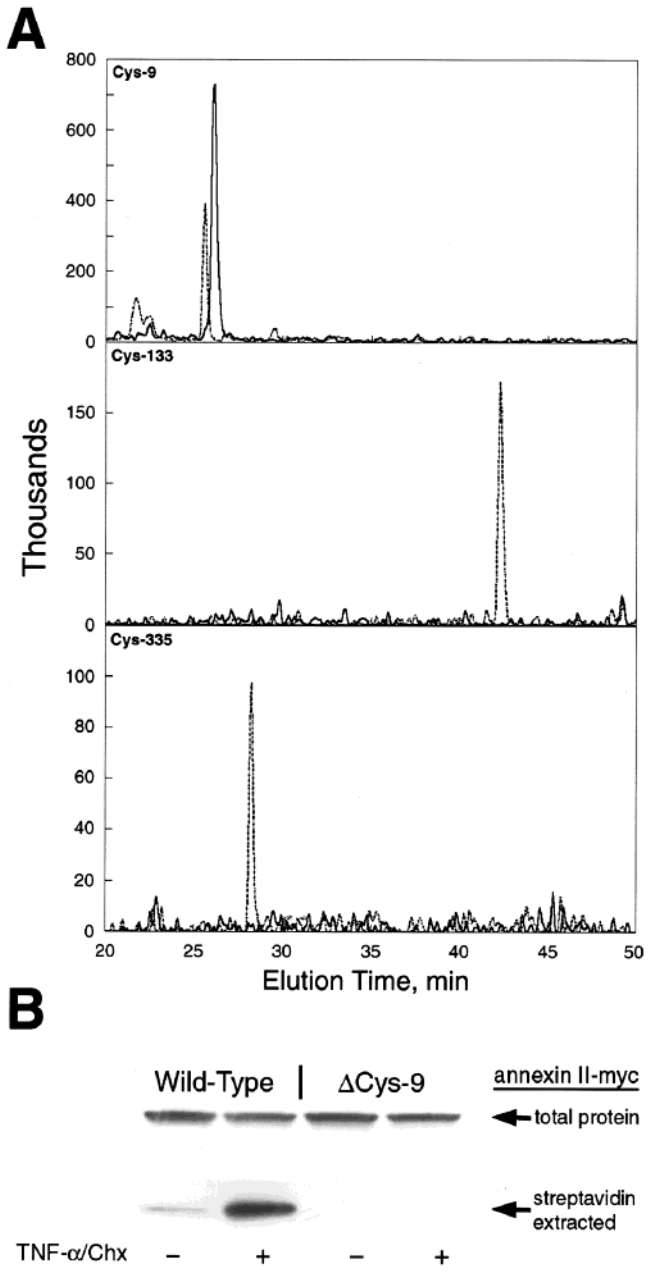

FIGURE 7: Identification of the reactive cysteine in annexin II. Panel A: Fifteen micrograms of bovine annexin II was reacted with 20 μM iodoacetamide at pH 6.0. Unreacted cysteines were alkylated with acrylamide, and the protein was digested with Lys-C. The peptides were then mapped by HPLC−mass spectrometry. The extracted ion chromatographs for both the iodoacetamide (solid line) and acrylamide (dashed line) derivatized peptides are shown for the three cysteine-containing peptides recovered from the digest. Of these, only the Cys-9-containing peptide shows significant derivatization with iodoacetamide. Panel B: HeLa cells transfected with myc-tagged wild-type or Cys-9 mutated annexin II cDNAs were treated with BioGEE alone or BioGEE in the presence of 10 ng/mL TNF-α and 1 μg/mL Chx for 4 h. Biotin-containing proteins were extracted from 0.5 mg of soluble protein as described in Figure 1 and Experimental Procedures. Proteins eluting with DTT were resolved by SDS−PAGE and transferred to nitrocellulose for western blotting with antibodies against the myc epitope tag.

DISCUSSION

It has become increasingly apparent that reactive oxygen species are involved in a variety of physiological processes. Despite this, the nature of the molecular mechanisms by which physiological responses to oxidants are mediated remains unclear. Oxidation of protein cysteinyl thiols, with a protein−glutathione mixed disulfide intermediate, is an attractive candidate regulatory mechanism for a number of reasons. The rapid formation of protein−glutathione adducts in cells exposed to oxidant stress was demonstrated in the 1980s (*19*), and several proteins were subsequently shown to be sensitive to thiol disulfide exchange reactions in vitro (*20*). In addition, the functioning of some proteins has been shown to be reversibly altered as a consequence of cysteinyl thiol oxidation to mixed disulfides (*11*, *21*−*23*). In vivo, protein S-glutathiolation could result from thiol disulfide exchange reactions involving oxidized glutathione or from direct oxidation of protein cysteinyl thiols followed by reaction with reduced glutathione. In either case, specificity could be generated from protein secondary and tertiary structure by promoting the ionization of an exposed cysteinyl thiol to a relatively nucleophilic thiolate anion. These reactive cysteine residues could be significantly oxidized at ROS concentrations below that which induces irreversible damage to the cell. Once the oxidant is removed from the system,

reversal of protein−glutathione mixed disulfides could be achieved by one of at least three enzymes (i.e., thioredoxin, glutaredoxin, or protein disulfide isomerase) to restore the protein to its basal, reduced state. Therefore, protein cysteinyl thiol oxidation involving protein−glutathione mixed disulfides contains, in theory, all of the elements of a regulatory system: sensitivity, specificity, and reversibility.

One impediment to firmly establishing cysteine oxidation−reduction as a physiological regulatory mechanism has been the failure of established analytical techniques to provide more than cursory information about the targets of ROS in the intact cell. To this point, incorporation of L-[^{35}S]cysteine into low molecular weight thiols has been the only technique available for specifically labeling S-glutathiolated proteins in situ. Although this approach has yielded a considerable amount of useful information, it has a number of drawbacks. First, labeling of the glutathione pool must be carried out in the presence of a high concentration of cycloheximide to prevent incorporation of the label directly into protein; therefore, all of the data obtained using this method come from cells that would be expected have substantial perturbations of many physiological processes due to depletion of proteins with short half-lives. Second, L-[^{35}S]cysteine labeling provides little information about the individual proteins being oxidized beyond molecular weight. Finally, isolation of S-glutathiolated proteins labeled using L-[^{35}S]cysteine can only be accomplished using traditional protein purification methods and has, thus far, yielded definitive identification of only a few proteins. To try to address some of the limitations of L-[^{35}S]cysteine, an alternative approach was developed using a cell-permeant glutathione analogue. Because the approach described allows for selective identification of proteins that have biotin incorporated through a disulfide linkage, a modification that is not known to occur naturally, it is not necessary to inhibit protein synthesis or perturb the cell in any way. Also, as demonstrated with TPxII and annexin II, the protocol allows for the rapid purification of S-glutathiolated proteins in sufficient quantity for peptide mapping or sequencing, thus allowing for definitive identification.

Because most cells do not take up glutathione, a biotinyl moiety was added at the primary amino group of glutathione ethyl ester, which has been demonstrated to efficiently cross the plasma membrane (*16*). Two of the proteins identified using BioGEE, GAPDH and TPxII, are thought to be exclusively intracellular, and no labeling of proteins occurred when BioGEE was added to the medium just prior to lysing the cells. These findings indicate that the label is being incorporated into intracellular proteins prior to cell lysis and, therefore, that the addition of biotin to the amino terminus of glutathione ethyl ester does not prevent its crossing the plasma membrane.

Studies done with glutathione ethyl ester indicate that it is rapidly converted to glutathione by esterases once it is inside the cell (*16*). This is probably true for BioGEE as well, although this remains to be confirmed. It is conceivable, however, that the remaining biotinyl moiety could affect the ability of enzymes such as glutaredoxin to use biotinylated glutathione as a substrate. If that were the case, BioGEE might be expected to have a slower off rate and therefore accumulate in protein to a greater extent than endogenous glutathione. Likewise, the extent of BioGEE incorporation into protein is dependent upon the unknown ratio of BioGEE to endogenous glutathione in the cell. It should therefore be stressed that the extent to which BioGEE is incorporated into a given protein cannot at this point be directly correlated with the extent to which that protein is glutathiolated. The findings presented here instead indicate that incorporation of BioGEE into protein is a valid marker for the presence of oxidant-modified cysteines and can be used to identify both protein targets of oxidant stress within the cell and the oxidant-modified cysteines within a given protein.

To identify proteins that might be regulated by S-glutathiolation in conjunction with a physiological process, BioGEE was used to purify proteins from cells undergoing TNF-α-induced apoptosis. There is a considerable amount of data indicating that oxidants are involved in signal transduction by a variety of proapoptotic stimuli (*24*−*26*). In HeLa cells, TNF-α administered with cycloheximide induces a slow rise in cell-associated reactive oxygen species that is required for induction of apoptosis (*18*). Treatment of HeLa cells with TNF-α and cycloheximide was found to induce a significant increase in the amount of several proteins bound to BioGEE. This finding is important because it indicates that HeLa cells are capable of generating sufficient oxidant stress to induce protein S-glutathiolation, a prerequisite for agonist-induced regulation of protein function by S-glutathiolation.

Peptide mapping and sequence analysis of proteins in the streptavidin extract revealed that TPxII and annexin II are S-glutathiolated in association with TNF-α-induced apoptosis. This finding, and the oxidant-dependent incorporation of BioGEE, was confirmed in western blots. These data indicate that S-glutathiolation of proteins as a consequence of direct H_2O_2 challenge is similar to S-glutathiolation induced by a physiological agonist and is consistent with data indicating that H_2O_2 mediates many redox-dependent responses. The functional consequences of TPxII and annexin II S-glutathiolation remain to be determined. It is believed that a reactive cysteine in the active site of TPxII provides electrons for reduction of hydroperoxides; however, the discovery of an S-glutathiolated intermediate was unexpected since a recent study found that TPxII could not use reducing equivalents supplied by glutaredoxin for its peroxidase activity (*27*). This raises the possibility that TPxII contains a second redox-active cysteine, outside of its active site, that might be involved in regulation of the peroxidase or other functions. In this regard it is intriguing to note that TPxII has recently been shown to regulate the cytostatic activity of c-Abl in a redox-dependent fashion through direct protein−protein association (*28*).

Evidence for the existence of a redox-sensitive cysteine in annexin II is consistent with a previous study showing that Cys-9, a cysteine in the N-terminal domain of annexin II, could form a mixed disulfide with homocysteine (*29*). In that case, formation of the mixed disulfide prevented binding of tissue plasminogen activator to annexin II on the outer leaflet of the endothelial cell membrane. The results obtained with BioGEE indicate a more global reactivity of Cys-9 and suggest that a variety of oxidant stresses, including H_2O_2 and superoxide, could interfere with tissue plasminogen activator binding to annexin II.

Our results obtained with TNF-α demonstrate that endogenously produced ROS have the capacity to induce S-

glutathiolation of a handful of specific proteins. Although establishing the exact physiological consequences of S-glutathiolation awaits further study, the fact that a physiological oxidative stress can specifically alter the posttranslational state of a set of target proteins suggests a mechanism by which diffusible, reactive molecules such as H_2O_2 can function as specific signaling molecules in cells. As such, the further identification of S-glutathiolated proteins should help to define the specific signaling pathways whose activities are physiologically regulated by intracellular oxidants.

ACKNOWLEDGMENT

The authors thank Dr. Henry Fales (NIH) for valuable assistance in the preparation of BioGEE.

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BI0007674