Oxidative modification of H-ras: S-thiolation and S-nitrosylation of reactive cysteines

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The reactive cysteines in H-ras are subject to oxidative modifications that potentially alter the cellular function of this protein. In this study, purified H-ras was modified by thiol oxidants such as hydrogen peroxide (H₂O₂), S-nitrosoglutathione, diamide, glutathione disulphide (GSSG) and cystamine, producing as many as four charge-isomeric forms of the protein. These results suggest that all four reactive cysteines of H-ras are potential sites of regulatory modification reactions. S-nitrosylated and S-glutathiolated forms of H-ras were identified by protocols that depend on separation of alkylated proteins on electrofocusing gels. S-nitrosoglutathione could S-nitrosylate H-ras on four cysteine residues, while reduced glutathione (GSH) and H₂O₂ mediate S-glutathiolation on at least one cysteine of H-ras. Either GSSG or diamide S-glutathiolated at least two cysteine residues of purified H-ras. Iodoacetic acid reacts with three cysteine residues. In intact NIH-3T3 cells, wild-type H-ras was S-

glutathiolated by diamide. Similarly, cells expressing a C118S mutant or a C181S/C184S double mutant of H-ras were S-glutathiolated by diamide. These results suggest that H-ras can be S-glutathiolated on multiple thiols *in vivo* and that at least one of these thiols is normally lipid-modified. In cells treated with S-nitrosocysteine, evidence for both S-nitrosylated and S-glutathiolated H-ras was obtained and S-nitrosylation was the predominant modification. These results show that oxidative modification of H-ras can be extensive *in vivo*, that both S-nitrosylated and S-glutathiolated forms may be important, and that oxidation may occur on reactive cysteines that are normally targeted for lipid-modification reactions.

Key words: glutathione, MAP kinase, protein oxidation, S-nitroso cysteine.

INTRODUCTION

H-ras is a small guanine nucleotide binding protein (G-protein) that is essential for cellular proliferation and differentiation [1]. It has recently been implicated both as an initiator of oxidant-related signalling [2,3] and as a target for oxidative regulation [4–7]. It has been suggested that oxidative signals are relayed to the extracellular signal-regulated kinase (ERK) pathway through H-ras [4–7]. Hydrogen peroxide, nitric oxide (NO), superoxide, sodium nitroprusside and S-nitroso-N-acetylpenicillamine activated ERK and other components of the ERK pathway in intact cells [4–7]. A direct modification of Cys-118 by NO may be responsible for the activation of H-ras in a T-cell line, and S-nitrosylation of H-ras may be at least partially responsible for the proliferative effects of NO in some cell lines [8,9]. Because Cys-118 resides on a loop that has contact with the guanine nucleotide, it has been suggested that modification of this cysteine may directly affect the bound GTP/GDP ratio of H-ras, thus changing its activity [10].

H-ras possesses three other cysteine residues in addition to Cys-118, that may be oxidatively modified *in vivo*. The modification of any of the other cysteines can actually oppose the activation of H-ras through Cys-118 modification, since the other cysteines are involved in membrane localization of the protein. Thus, Cys-181 and Cys-184 of H-ras are palmitoylated *in vivo* [11,12] and the most C-terminal cysteine, Cys-186, is farnesylated [11,13–15]. Since palmitoylation of cysteine involves

a thioester linkage, it may be susceptible to thiol-mediated modification by simple exchange with low-molecular-mass thiols such as glutathione. On the other hand, the farnesyl group is linked by a more inert thioether. It has been suggested that from 10% to 50% of the H-ras in cells may not be associated with membranes [12,16]. This fraction is potentially not lipidated.

Recently, it has been reported that the palmitates on H-ras undergo significant metabolic turnover *in vivo*, and, under conditions of nitrosative stress, the turnover is increased [17]. In addition, reports have suggested that NO and NO-producing oxidants can prevent palmitoylation of some proteins in neuronal cells [18]. These effects may result from direct modification of Cys-181/184/186 by nitrosating agents such as S-nitrosocysteine [19] or the regulation of some as yet uncharacterized enzyme involved in palmitate metabolism. Mutagenesis studies have previously confirmed that Cys-181/184/186 are essential for transformation of cells by an otherwise oncogenic H-ras mutant form [12], and the effect of S-nitrosocysteine on the palmitates of H-ras is correlated with deactivation of the ERK pathway [17].

Because of the abundance of free reduced glutathione (GSH) in cells, any mechanism which suggests that a protein is S-nitrosylated in cells must also address the participation of GSH in oxidative events [20]. We have recently shown that S-nitrosocysteine produces significant S-nitrosylation of total protein thiols in cells as well as a lesser amount of S-glutathiolation and S-cysteinylation [19]. Different sites on proteins are likely to exhibit widely different reactivities for oxidants [21]. Similarly, it

Abbreviations used: Ab, antibody; BCS, bovine calf serum; DTT, dithiothreitol; ERK, extracellular signal-regulated kinase; GSNO, S-nitrosoglutathione; IAA, iodoacetic acid; IAM, iodoacetamide; IEF, isoelectric focusing; NEM, N-ethylmaleimide; GSH refers only to the reduced form of glutathione. GSSG refers only to glutathione disulphide. Oxidized glutathione may refer to several forms of glutathione including GSSG, cysteine-glutathione disulphide, S-glutathiolated protein and S-nitrosoglutathione.

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is likely that different oxidant-related adducts would produce different effects because of size and charge-related issues. It is important, therefore, to differentiate between the several possible end-points of oxidative events in cells. While previous studies have suggested that H-ras may be activated [4–9] or inactivated [17] through *S*-nitrosylation or oxidation of critical cysteine residues, there have been no systematic studies to date to isolate the products of these oxidative reactions. For this reason it is important to develop means to identify and characterize the oxidation of the H-ras cysteines, both using the purified protein and also in cells. This paper provides evidence that H-ras can be oxidatively modified simultaneously on four separate cysteine residues *in vitro*. We further show that while *S*-nitrosylation may modify all four of these residues, *S*-glutathiolation occurs primarily on two of four possible reactive cysteine residues. We provide evidence that one or more sites are available to *S*-glutathiolation *in vivo*, and that at least one of these sites is a cysteine normally required for lipidation of H-ras. Furthermore, H-ras can be both *S*-nitrosylated and *S*-glutathiolated more efficiently than most cytosolic proteins within NIH-3T3 cells treated with *S*-nitrosocysteine.

MATERIALS AND METHODS

Materials

Iodoacetic acid (IAA), iodoacetamide (IAM), *N*-ethylmaleimide (NEM), reduced glutathione (GSH), dithiothreitol (DTT), glutathione disulphide (GSSG), rat anti-mouse alkaline phosphatase-conjugated antibody and goat anti-rat alkaline phosphatase-conjugated antibody were from Sigma (St. Louis, MO, U.S.A.). Cell culture reagents were purchased from GIBCO-BRL (Gaithersburg, MD, U.S.A.) with exception of bovine calf serum (BCS), which was purchased from HyClone (Logan, UT, U.S.A.). Ampholytes were purchased from Amersham-Pharmacia, Inc (Piscataway, NJ, U.S.A.). Recombinant human H-ras and agarose-conjugated and soluble antibody-1 (Ab-I) were purchased from Calbiochem-Novabiochem (San Diego, CA, U.S.A.). Monoclonal Ab 146-03E4 (Ab 146) was purchased from Quality Biotech (Camden, NJ, U.S.A.).

Preparation of H-ras protein

Recombinant human wild-type H-ras (3 mg/ml) was completely reduced with 10 mM DTT for 30 min at room temperature. The protein was then dialysed for 24 h at 4 °C against 20 mM Tris/HCl, pH 8.0, containing 500 μ M DTT. DTT was included in the dialysis buffer to prevent the progressive oxidation of H-ras that occurred in the absence of DTT (results not shown). Dialysed protein (approx. 1 mg/ml) was stored for less than 1 week at 4 °C before use. Protein concentration was determined as described by Lowry et al. [22].

Preparation of *S*-nitrosothiols

S-nitrosoglutathione was prepared as previously described in [23] and used immediately after preparation. Briefly, 220 μ l each of 220 mM GSH and 220 mM sodium nitrite were mixed with 25 μ l of 4.0 M HCl and incubated in the dark at room temperature for 10 min. The solution was then neutralized with 25 μ l of 4.0 M NaOH to give a final concentration of approx. 100 mM *S*-nitrosoglutathione. The final concentration was calculated from absorbance at 334 nm using the absorption coefficient $767 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [24].

Isoelectric focusing (IEF) of H-ras

Purified H-ras was separated on horizontal slab gels (5.0 % acrylamide/2.7 % Bis-acrylamide/1 % ampholyte pH 3.5–5.0/1 % ampholyte pH 4.0–6.0) at 1500 V and 0.5 mA/cm² for 50 min. Protein bands were stained with Coomassie Blue. Approx. 1 μ g of H-ras was applied to each lane.

Electrofocusing gels containing NetFix [23] were analysed by Western blot methods as follows. Gels were transferred to Immobilon-P (PVDF) (Millipore, Inc, Bedford, MA, U.S.A.) membrane using a Semi-Dry Transfer Cell (Bio-Rad Laboratories, Hercules, CA, U.S.A.). H-ras was visualized with the Ab 146 primary antibody (1:3000 dilution) and rat anti-mouse alkaline phosphatase secondary Ab (1:10000 dilution). The truncated mutant (Δ 167–189) was visualized with Ab-1 as the primary antibody (1:3000 dilution) and goat anti-rat secondary antibody (1:10000 dilution). Bands were visualized with *p*-Nitroblue Tetrazolium Chloride/5-bromo-4-chloro-3-indolyl phosphate.

Quantification of IEF gels

Gels were scanned and bands were quantified using Image Quant v3.3 (Molecular Dynamics Inc.). The extent of modification of H-ras was calculated by determining relative band densities from the completely reduced band of H-ras (band 1) to the most highly modified band of H-ras (band 4) within individual lanes. When the protein was alkylated with IAM, bands modified with a negatively charged adduct (*S*-glutathiolated bands) were more acidic than the unmodified protein. If alkylated with IAA, bands modified with a neutral adduct (*S*-nitrosylated bands) were more basic than the unmodified protein. The fractional modification of H-ras (mole cysteine modified per mole protein) was calculated as follows.

$$\begin{aligned} \text{Fractional modification} = & \text{density of band 2} \\ & (1 \text{ modified cysteine}) + \\ & 2 \times \text{density of band 3} \\ & (2 \text{ modified cysteines}) + \\ & 3 \times \text{density of band 4} \\ & (3 \text{ modified cysteines}) / \\ & \text{total density of all bands} \end{aligned}$$

Modification of H-ras in soluble extracts of *Escherichia coli* overexpressing H-ras

As described by Ji et al. [23], *E. coli* strain JM 105 containing the gene for H-ras wild-type, C118S/C181S/C184S or truncated (Δ 167–189) mutant inserted in a pAT vector [25] were grown at 37 °C in Luria broth to an attenuation of 1.2–1.5 (at 600 nm) and induced by the addition of isopropyl β -D-thiogalactopyranoside for 3 h. The cells were then centrifuged at 16000 *g* for 10 min. The pellet was washed with 20 mM Tris/HCl buffer (pH 7.2), 100 mM NaCl 5 mM MgCl₂ and 1 mM PMSF. After centrifugation at 16000 *g* for 10 min, cells were resuspended to 0.2 g of cell paste/ml in 20 mM β -glycerophosphate/HCl (pH 7.0) and sonicated for 30 s. The soluble fraction was obtained by ultracentrifugation in a Beckman Airfuge at 160000 *g* for 30 min.

Cell culture

NIH-3T3 fibroblasts were obtained from the American Type Culture Collection (ATCC) and cultured according to their recommendations (Dulbecco's Modified Eagle Medium + 2 mM glutamine + 0.2 mM sodium pyruvate + 10 % BCS + 100 units/

ml each of penicillin and streptomycin). NIH-3T3 cells overexpressing wild-type and C118S H-ras were gifts from L.A. Quilliam (Indiana University School of Medicine, Indianapolis, IN, U.S.A.). NIH-3T3 cells overexpressing C181S/C184S H-ras were the gift of B.M. Willumsen (University of Copenhagen, Copenhagen, Denmark). For all experiments, NIH-3T3 cells were grown to confluence (assessed visually, approx. $1-2 \times 10^6$ cells/35 mm plate) and the medium was changed. Experiments were started 24 h later by replacing medium with PBS. Cells were incubated in PBS for 5 min prior to starting an experiment.

Analysis of low-molecular-mass thiols in NIH-3T3 cells

Cells were rinsed twice with ice-cold PBS and lysed with 10% perchloric acid. Plates were scraped and the soluble and precipitated materials were collected and stored at -20°C for less than 1 week. Precipitated proteins were removed by centrifugation, and a fraction (200 μl) of the supernatant was used for the HPLC analysis of low-molecular-mass thiols. The pelleted material was stored at -20°C for protein determination.

Low-molecular-mass thiols were analysed as described by Fariss and Reed [26] with some modifications. Briefly, IAA was added to the 200 μl sample containing 10% perchloric acid to a final concentration of 130 mM and the pH was raised to ~ 8.5 by addition of dry potassium bicarbonate. An equal volume of fluorodinitrobenzene in 100% ethanol was added to a final concentration of 0.5% and incubated overnight at 4°C . Samples were separated on an aminopropyl anion-exchange column. Compounds were identified by co-elution with authentic pure compounds. In cells labelled with Tran ^{35}S -label (ICN Pharmaceuticals, Inc., Costa Mesa, CA, U.S.A.) the specific activities of GSH and GSSG were determined by collecting the peaks and counting radioactivity by liquid scintillation.

Assay of S-thiolated H-ras

S-thiolated H-ras was determined using a modification of the procedure of Fariss and Reed [26]. Briefly, H-ras was precipitated by adding 70% perchloric acid to a final concentration of 10%. After centrifugation at 15000 g, the supernatant was removed and used for quantification of GSSG/GSH by HPLC. The protein pellet was washed with ice-cold 100% ethanol, resuspended in 50 mM MOPS (pH 8.0) $\pm$ 50 mM DTT, and incubated for 60 min at 37°C . Perchloric acid was again added to a final concentration of 10% and the sample was centrifuged at 15000 g. The supernatant was recovered and thiols were analysed by anion-exchange HPLC as described above.

Immunoprecipitation of H-ras

Cells were washed twice with ice-cold PBS and lysed with immunoprecipitation buffer [Tris-buffered saline/1% Triton X-100/0.5% sodium deoxycholate/0.1% SDS] containing 50 mM NEM to prevent oxidative modification of proteins. Lysates were centrifuged at 15000 g and H-ras was immunoprecipitated with agarose-conjugated Ab-1 by incubating at 4°C for 2 h. The immunoprecipitation beads were first rinsed twice with immunoprecipitation buffer and then centrifuged twice through immunoprecipitation buffer containing 7.5% sucrose. Approx. 5 μl of beads were used per 0.5 mg of total protein to yield 0.1–0.2 μg of H-ras.

RESULTS

Reactive cysteines on H-ras

An SDS/PAGE gel of the H-ras protein used in this study shows a single band (Figure 1A), suggesting that the protein is at least 95% homogeneous, but IEF of the IAM-alkylated protein revealed a single acidic contaminating protein band (left lane in Figure 1B). Proteins with reactive cysteines, i.e. H-ras, must be alkylated to protect these cysteines during IEF. IAM is normally used, thereby producing uncharged alkylation products (Figure 1B). The contaminating band migrated at a more acidic pI, consistent with the presence of an acidic sulphhydryl oxidation product such as a sulphinic or sulphonic acid. This oxidized band was recognized by the H-Ras antibody in Western blotting experiments with both purified H-Ras (Figure 1C) and *E. coli* extracts (results not shown). It was not possible to eliminate this contaminating band even in freshly prepared protein, and subsequent experiments were performed on protein containing this contaminating protein band.

H-ras may be alkylated with IAA to generate negatively charged sites at reactive cysteines (right lane, Figure 1B). The IAA-alkylated H-ras protein in Figure 1(B) has one major and two minor bands that can be attributed to alkylation of three reactive cysteines on H-ras. Approx. 90% of the IAA-treated H-ras had three additional negative charges while 10% had two additional negative charges. Since there are four potentially reactive cysteines on H-ras, this experiment indicated that one of

Figure 1 Analysis of H-ras modification by electrofocusing

(A) Coomassie Blue stained SDS/PAGE. Commercially available H-ras was examined for contaminating protein bands after each sample was reduced and alkylated as described in the Materials and methods section. Positions of molecular-mass markers are indicated on the left. (B) Coomassie Blue stained IEF gel of H-ras. H-ras was examined for contaminating protein bands. Protein (10 μM) was reduced with 10 mM DTT for 30 min at pH 8.0 and 37°C , then diluted 2-fold and alkylated with either 20 mM IAM in 20 mM Tris/HCl buffer (pH 8.0) or 40 mM IAA in 60 mM Tris base (final pH = 7.5). One microgram of each derivatized protein was applied to each lane by published procedures as described in the Materials and methods section. (C) Western blot analysis of an IEF gel of pure H-ras and soluble protein extracts from *E. coli*. Proteins were analysed after alkylation with either IAM or IAA as appropriate. *E. coli* overexpressing either the wild-type or a single mutant form of H-ras were cultured and lysed as described in the Materials and methods section. Before separating samples on IEF gels, proteins were alkylated either with 40 mM IAM or with 40 mM IAA at pH 7.5 for 15 min. The contaminating acidic band normally found in the expressed wild-type protein was not apparent in this experiment. WT, wild type.

Figure 2 Electrostatic surface of H-ras

An electrostatic surface calculation of the surface of H-ras with a central focus on Cys-118 (labelled in figure) is shown. The X-ray crystal structure of H-ras (PDB Accession code 121P) was used for calculations with MolMol [44]. Positively charged areas are black, neutral regions are grey and negatively charged regions are white. The positive region nearest Cys-118 is marked with '+' while the nearest negative region is highlighted with '-'.

these might be less reactive with IAA. It should be noted that the negative charge of IAA might limit its ability to alkylate protein cysteines in regions of the protein with high negative charge. To determine which cysteines in H-ras were reactive with IAA, wild-type and mutant forms of H-ras were expressed in *E. coli* and analysed by electrofocusing after IAA treatment (Figure 1C). Pure H-ras and the wild-type expressed protein from *E. coli* lysates gave similar results (lanes 1–4). The IAM-treated reduced form of the protein had a pI of 4.3 (lanes 1 and 3) and the IAA-alkylated form of the protein had a pI of 3.9 (lanes 2 and 4). This result suggests that three cysteines reacted with IAA in this experiment, similar to the experiment illustrated in Figure 1(B). When C118/181/184S H-ras was compared with wild-type H-ras, the reduced form had a pI of 4.3 when alkylated with IAM (lane 5) and 4.1 when alkylated with IAA (lane 6). These results are consistent with a single IAA alkylation site on this modified protein, i.e. Cys-186. Expression of a truncated form of H-ras ($\Delta 167$ –189) produced a protein with a pI of 4.1 that was unreactive to IAA (note that lanes 7 and 8 have the same pI). Since the truncated H-ras contains only Cys-118, the result suggests that IAA cannot readily react with that cysteine. The pI of the truncated H-ras was 4.1, reflecting the loss of a single positive charge associated with the 167–189 segment of H-ras. This experiment identifies the three IAA-reactive cysteines of H-ras as Cys-181, Cys-184 and Cys-186. Cys-118, which is surface exposed in X-ray crystal structures and NMR structures of H-ras [10,27], does not appear to be reactive towards IAA. Figure 2 shows that an electrostatic surface diagram of H-ras confirms the presence of extensive negative charge around Cys-118. Thus, the charge of the protein may inhibit reaction of Cys-118 with IAA.

S-glutathiolated forms of H-ras

Potentially, S-glutathiolation is an important modification of H-ras resulting from either oxidative or nitrosative stress [19]. A simple test for S-glutathiolation of H-ras cysteines can be accomplished by a thiol-disulphide exchange reaction with the negatively charged GSSG. Although this reaction may be a minor mechanism for S-glutathiolation in intact cells [28], it is

Figure 3 S-glutathiolation of H-ras with GSSG

H-ras (10 μ M) was incubated with 20 mM GSSG for the indicated times at 37 °C and pH 8.0. The reactions were stopped by alkylation with IAM. The alkylation and reduction of the protein were as described in Figure 1(B). In lane 3 the protein was treated with 10 mM IAM at pH 8.0 for 5 min before adding GSSG. RX, reaction.

Figure 4 S-glutathiolation of H-ras with hydrogen peroxide

(A) H-ras (10 μ M) was incubated with 2 mM H_2O_2 + 3 mM GSH (lanes 3 and 4) or 1.5 mM GSSG (lanes 5 and 6) for 5 min at 37 °C and pH 8.0. The reactions were stopped by alkylation with IAM. The alkylation and reduction of the protein were as described in Figure 1(B).

easily controlled *in vitro* and it does not produce undesirable side reactions. In Figure 3, lanes 1 and 2 identify the pI values of the reduced IAM- and IAA-treated protein. As discussed in the previous section, IAM-treated protein bands have no charged ligands on the sulphhydryls (pI 4.3) and IAA-treated bands can have up to three additional negative charges (pI 3.9). Lane 3 shows that the IAM-alkylated protein did not react with added GSSG, i.e. there were no acidic protein forms as expected from S-glutathiolation with the negatively charged glutathione molecule. Thus, IAM effectively prevents any reaction with GSSG. The time course of reaction with GSSG (lanes 4–6) shows that two cysteines react readily, while three and possibly even four cysteines (lane 6) can react with GSSG. Lane 7 shows that reduction of the protein in lane 6 with DTT removes the glutathione bound to the S-glutathiolated sites, thereby removing the negative charge associated with the disulphide-attached glutathione. Apparently the pI values of the partially S-glutathiolated forms are the same as the IAA-alkylated forms demonstrated in Figure 1. Thus, addition of a single negative charge to the protein produced a band at pI 4.1, a second charge gave the band at 4.0, and a third gave the band at 3.9.

A more useful model for the oxidative S-glutathiolation of H-ras in intact cells is illustrated by an experiment shown in Figure 4. When hydrogen peroxide was added to H-ras in the presence of 3 mM GSH, acidic protein bands (lane 3) that were susceptible to reduction with DTT (lane 4) were formed. S-glutathiolation occurs by reaction of hydrogen peroxide with protein sulphhydryls, thereby producing a partially oxidized protein sulphhydryl derivative that can subsequently combine with GSH to yield an S-glutathiolated protein [28]. When H-ras was incubated with hydrogen peroxide in the absence of glutathione, irreversibly oxidized forms of the protein were generated (results not shown).

Figure 5 Reaction of H-ras with S-nitrosoglutathione

(A) H-ras ($10 \mu\text{M}$) was incubated with 5 mM S-nitrosoglutathione for 5 min at 37°C and pH 8.0. The reaction was stopped by alkylation with IAM (lanes 1 and 2) or IAA (lanes 4 and 5) as described in Figure 1(B). The protein was reduced (lanes 3 and 6) as described in Figure 1(B) and alkylated with either IAM (lane 3) or IAA (lane 6). (B) H-ras ($10 \mu\text{M}$) was incubated with 5 mM cystamine for the indicated times at 37°C and pH 8.0. The reactions were stopped by alkylation with IAM. The alkylation and reduction of the protein were as described in Figure 1(B). (C) *E. coli* overexpressing truncated H-ras were cultured and lysed as described in the Materials and methods section. Extracts were then treated with 5 mM S-nitrosoglutathione for 15 min and the reaction was stopped with 20 mM cystamine at pH 7.5. One sample (lane 4) was reduced with 10 mM DTT for 15 min before adding cystamine. Extracts were separated and visualized by IEF/Western blot analysis as described in the Materials and methods section.

In this case negatively charged forms of H-ras probably result from oxidation of cysteines to either sulphinic or sulphonic acids. These forms are considered 'irreversibly oxidized' because there is no known cellular mechanism for reduction of these products.

GSSG was potentially generated by the action of hydrogen peroxide on the glutathione pool, producing a pool of glutathione that could initiate S-glutathiolation of H-ras by thiol-disulfide exchange. To eliminate this possibility, H-ras was incubated with appropriate amounts of GSSG (Figure 4, lanes 5 and 6). If all the glutathione were oxidized by hydrogen peroxide, 1.5 mM GSSG would be produced. H-ras incubated with 1.5 mM GSSG for the duration of the experiment did not contain significant amounts of acidic protein forms (lanes 5 and 6). Thus, S-glutathiolation of H-ras did not occur via a GSSG intermediate (lane 3). In addition, the concentrations of both GSH and GSSG were determined at the conclusion of the reaction. The sample shown in lane 3 contained 0.9 mM GSH and 1.05 mM GSSG, suggesting that hydrogen peroxide did not completely oxidize the glutathione pool.

The hydrogen peroxide-initiated S-glutathiolation of H-ras as observed by gel electrofocusing was compared with the direct determination of the protein-bound glutathione. An average of 0.75 mol cysteine per mol H-ras were associated with the hydrogen peroxide-catalysed S-glutathiolation reaction whether measured by electrofocusing gels or by reductive release of

protein-bound glutathione. Thus, S-glutathiolation accounted for the acidic H-ras bands observed by electrofocusing.

S-Nitrosylation of H-ras by transnitrosation

S-nitrosylation has been increasingly recognized as an important oxidative modification of proteins with reactive cysteines [23,29–31]. A truncated form of H-ras (containing only the reactive Cys-118) has been shown to react with NO [8], and we recently demonstrated that wild-type H-ras could be S-nitrosylated on at least three cysteines [23]. Since H-ras may react with NO *in vivo* and be either activated [8] or inactivated [17], it seemed that there could be multiple sites of modification with NO, perhaps different sites driving the activation or inactivation. We therefore examined the site-specific S-nitrosylation of H-ras by transnitrosation with S-nitrosoglutathione (GSNO). Since the GSNO potentially generates both S-glutathiolated and S-nitrosylated H-ras, the reaction was analysed for both forms of protein modification. Figure 5(A) shows reactions with pure recombinant H-ras. The protein was alkylated with IAM (see lanes 1–3), to determine if S-glutathiolated forms (negatively charged) of H-ras were generated, and was alkylated with IAA (lanes 4–6) to visualize uncharged forms of H-ras, i.e. S-nitrosylated forms. Since IAA did not react with Cys-118 (Figure 1C), this method was applicable to three of the H-ras cysteines, i.e. Cys-181, Cys-184 and Cys-186. There was limited, but significant, S-glutathiolation of H-ras, as demonstrated by the increase in the pI 4.1 band in lane 2. As expected, this modification was reversed by DTT (lane 3). On the other hand, lane 5 demonstrates extensive S-nitrosylation of all three reactive cysteines on the carboxyl tail of H-ras (note that S-nitrosylated protein bands migrate with a higher pI because S-nitrosylation prevents the alkylation with negatively charged IAA). Again, this modification was reversed by reduction with DTT (lane 6). We confirmed that Cys-181, Cys-184, and Cys-186 were the reactive cysteines that were nitrosylated by testing the reaction of the C118/181/184S H-ras with GSNO. We found only one NO reactive site on this protein corresponding to a reaction with the only reactive cysteine remaining, i.e. Cys-186 (results not shown).

S-nitrosylation of Cys-118 could only be detected with a different sulphhydryl-modifying agent. Using the positively charged disulphide, cystamine, it was possible to demonstrate S-nitrosylation as shown in Figure 5(B). The reaction of H-ras with cystamine by thiol-disulfide exchange is very rapid and it produces a positively charged sulphhydryl adduct. As the reaction went to completion, two more positively charged protein forms were produced, suggesting that two cysteines were rapidly modified by this reagent. To confirm that Cys-118 was one of these sites, a truncated form of H-ras that did not contain Cys-181, Cys-184 or cys-186 was expressed in *E. coli*, modified with GSNO, and then analysed by Western blots/electrofocusing. Figure 5(C) shows that a single site on the unmodified expressed protein reacted with cystamine, producing a single more positively charged form of H-ras (lane 2). When the truncated H-ras was incubated with GSNO, modification by cystamine was blocked (lane 3). This experiment confirms the previously reported S-nitrosylation of Cys-118 in H-ras [8].

S-thiolation of H-ras in diamide-treated NIH-3T3 cells

In vivo, lipids modify all three cysteines on the C-terminus of H-ras (Cys-186 is prenylated, and Cys-181 and 184 are palmitoylated). To examine the availability of these cysteines for S-thiolation *in vivo*, NIH-3T3 cells expressing either wild-type C118S, or C181S/C184S H-ras were incubated with Tran-S³⁵-label to label the cellular glutathione pool, and then the cells were

Figure 6 Diamide-catalysed S-glutathiolation of H-ras in NIH-3T3 cells

NIH-3T3 cells overexpressing wild-type, C118S, or C181S/C184S H-ras were labelled with Tran³⁵S-label for 4 h in the presence of cycloheximide (100 μ g/ml), then treated with 2 mM diamide for 10 min. H-ras was immunoprecipitated as described in the Materials and methods section, and released from the immunoprecipitation beads by boiling for 5 min in SDS/PAGE sample buffer containing 10 mM NEM. NEM-modified proteins were separated by SDS/PAGE as previously described [45]. Replicate lanes were treated with 10 mM DTT before NEM treatment to release reduction-sensitive radioactivity. Gels were stained with Coomassie Blue, dried and exposed to film. Both radioactive and Coomassie Blue stained bands were quantified using densitometry and all radioactivity measurements were normalized to the same protein content. The background radioactivity detected in the DTT-treated samples was set equal to 1.

oxidized with diamide. Diamide produces extensive and reversible S-thiolation *in vivo* [32,33]. We confirmed that diamide could catalyse S-glutathiolation of pure H-ras by adding diamide to a suitable mixture of H-ras and glutathione *in vitro*. IEF gels of diamide-treated H-ras (not shown) were indistinguishable from those of GSSG-treated H-ras (Figure 3).

In a preliminary experiment, it was found that 2 mM diamide produced maximal S-thiolation of NIH-3T3 cell proteins, as assessed by incorporation of ³⁵S-labelled glutathione into the total protein pool. In cells expressing wild type, C118S or C181S/C184S, approx. 5 nmol adduct (³⁵S-glutathione) were bound to each milligram of total cellular protein by 2 mM diamide (results not shown). Figure 6 shows the effect of diamide on H-ras. The H-ras was immunoprecipitated from cells and S-glutathiolation of the recovered H-ras was studied. The top of Figure 6 shows the Coomassie Blue stained bands of the immunoprecipitated H-ras and the bottom shows the autoradiograph of the radioactivity associated with the bands. There was an easily detectable amount of S-glutathiolation in cells expressing either wild-type (lanes 1 and 2), C118S (lanes 3 and 4) or C181S/C184S (lanes 5 and 6) H-ras. Reduction with DTT nearly eliminated the radioactivity from the H-ras bands (compare lanes 1, 3 and 5 with lanes 2, 4 and 6 respectively), indicating that H-ras was S-glutathiolated under these conditions. There was no evidence for S-glutathiolation of wild-type H-ras in cells without diamide (results not shown). In addition, the possibility that contaminating radioactivity derived from either endogenous wild-type H-Ras or an antibody-precipitated, co-migrating protein unrelated to H-Ras was considered. An equivalent number of plates of NIH-3T3 cells expressing only endogenous H-Ras contained less than 1% of the radioactivity at the appropriate molecular mass on SDS/PAGE when compared with immunoprecipitated wild-type and C118S H-Ras from overexpressing 3T3 cells on the same gel (results not shown). Since the radioactivity shown in Figure 6 was only present in cells overexpressing some form of H-ras, endogenous H-ras did not contribute to the protein band density. Cys-186 was the only reactive cysteine present in all of the overexpressed proteins, suggesting that Cys-186 may be a primary site of S-thiolation *in vivo*. However, other cysteines cannot be ruled out, since each protein construct contained at least one other reactive cysteine.

Figure 7 Effect of S-nitrosocysteine of NIH-3T3 cells overexpressing wild-type H-ras

(A) The effect of 1 mM S-nitrosocysteine on low-molecular-mass thiols in NIH-3T3 cells was determined by anion-exchange HPLC as described in the Materials and methods section. (B) The total S-cysteinylation, S-glutathiolation and S-nitrosylation of the soluble protein pool was determined after cells were treated with 1 mM S-nitrosocysteine for the indicated times. Cultures were lysed with 10 mM β -glycerophosphate buffer containing 50 mM NEM. Protein S-thiolation was measured by anion-exchange HPLC of DTT-releasable low-molecular-mass thiols from the total cytoplasmic proteins as described in the Materials and methods section. Protein S-nitrosylation was measured by the nitrite released from dialysed soluble proteins as described in [1].

S-nitrosylation and S-glutathiolation of H-ras in S-nitrosocysteine-treated NIH-3T3 cells

S-Nitrosothiols such as S-nitrosocysteine have been used to simulate conditions that might cause protein S-nitrosylation in intact cells [19,34,35]. In addition, it was recently found that S-nitrosocysteine effectively modified the dynamics of H-ras palmitoylation [17], suggesting that modification of Cys-181 and/or Cys-184 might be affected by this reagent. Finally, it has been reported that S-nitrosocysteine causes S-nitrosylation, S-glutathiolation and S-cysteinylation of both intracellular glutathione and protein thiols in normal NIH-3T3 cells [19]. These experiments, taken together, suggest that either S-nitrosylation or S-glutathiolation (or both) might be responsible for redox regulation and modified lipidation of H-ras.

Because overexpression of H-ras can have profound effects on the cellular morphology and metabolism [12], the effect of S-nitrosocysteine was compared in normal and NIH-3T3 cells overexpressing wild-type H-ras. In spite of the fact that NIH-3T3/wild-type cells grew to a much higher population density with approximately three times the total protein of the normal NIH-3T3 cells, the GSH concentration per milligram of total cellular protein was similar to that of the parental NIH-3T3 cells. Figure 7(A) shows the effect of S-nitrosocysteine on the cysteine and glutathione pools of overexpressing cells. The changes mimic those recently reported for normal NIH-3T3 cells [19]. S-nitrosocysteine caused a rapid increase in S-nitrosoglutathione, cysteine, cystine and cysteine-glutathione disulphide while GSH

Table 1 *S*-nitrosylation and *S*-glutathiolation of H-Ras by *S*-nitrosocysteine

NIH-3T3 cells were incubated with PBS or 1 mM *S*-nitrosocysteine for 3 min and H-ras was immunoprecipitated from the extracts as described in the Materials and methods section. *S*-nitrosylation of H-ras. Duplicate samples of isolated H-Ras-agarose complex were rinsed once with detergent-free PBS to remove detergents that interfered with the nitrite assay. Protein-bound NO was released from the beads and determined as nitrite by the method described in [19]. The protein content of each sample was determined from the density of the H-ras band on SDS/PAGE. Samples were compared with a dilution series of purified H-Ras on the same gel (not shown). Approx. 1.2 µg of H-Ras was obtained for this assay from cells overexpressing H-Ras, but H-Ras was not detectable in the parental cell line. *S*-glutathiolation of H-Ras. NIH-3T3/WT cells were labelled with Tran³⁵S-label for 24 h as described in the Materials and methods section and subsequently treated with PBS or 1 mM *S*-nitrosocysteine for 3 min. *S*-glutathiolated H-Ras was determined from isolated H-Ras-agarose immunoprecipitates as described in the Materials and methods section. Since the protein-bound glutathione was ³⁵S-labelled, the amount bound was determined by liquid-scintillation counting of DTT-releasable soluble materials. The specific activity of cellular glutathione was obtained from identically treated cultures as described in the Materials and methods section. The specific activity of the glutathione was approximately 250 c.p.m./pmol. The protein content of each sample was determined from the density of the H-ras band on a Western blot of an SDS/PAGE gel. Samples were compared with a dilution series of purified H-Ras on the same gel (not shown). Approx. 0.3 µg of H-Ras was isolated for each duplicate determination. Autoradiography of the PVDF membrane after protein transfer revealed ³⁵S only in the H-Ras band. Thus no other protein contributed to the *S*-glutathiolation measured in this experiment. *n.d., Determination was below detectable limits for protein-bound NO (< 15 pmol/assay) or protein-bound glutathione (< 30 c.p.m./assay).

Experiment	Protein-bound NO (pmol/µg)	Protein-bound NO (pmol/pmol)	Protein-bound glutathione (pmol/µg)	Protein-bound glutathione (pmol/pmol)
NIH-3T3 parental cells				
No Cys-NO	n.d.*	n.d.	—	—
	n.d.	n.d.	—	—
NIH-3T3 + H-Ras				
No Cys-NO	n.d.	n.d.	n.d.	n.d.
	n.d.	n.d.	0.4	0.01
1 mM Cys-NO	53	1.1	3.3	0.07
	32	0.6	12.6	0.25

decreased. In addition, the effect of *S*-nitrosocysteine on protein *S*-thiolation and *S*-nitrosylation (Figure 7B) was similar to that reported for normal NIH-3T3 cells [19]. Protein *S*-nitrosylation was significantly more elevated than either protein *S*-glutathiolation or *S*-cysteylation. This experiment suggests that overexpression of H-ras does not significantly affect cellular responses to *S*-nitrosocysteine.

The oxidative effect of *S*-nitrosocysteine on immunoprecipitated H-ras is shown in Table 1. SDS/PAGE analysis showed that material precipitated by the agarose-gel method was primarily H-ras (results not shown). Control experiments in which immunoprecipitation beads were incubated with 5 mM GSNO (approx. 500 times the GSNO concentrations in cellular extracts) showed that non-specific association of GSNO with the beads did not occur (results not shown). Since GSNO is by far the most abundant *S*-nitrosothiol in *S*-nitrosocysteine-treated cells (Figure 7), any nitric oxide bound to the precipitated protein would have to be present as *S*-nitrosylated H-ras.

Table 1 provides no evidence of pre-existing *S*-nitrosylation of H-ras in untreated normal NIH-3T3 cells (top group of data), and in overexpressing cells similar results were obtained (second group of data). However, *S*-nitrosocysteine-treated cells contained an average of 43 nmol of *S*-nitrosothiol per milligram of H-ras (two determinations). This amount of *S*-nitrosylation is equivalent to 0.85 mol of *S*-nitrosothiol per mol of H-ras. The *S*-nitrosylation of H-ras was compared with the *S*-nitrosylation of the total soluble protein extract from these cells (Figure 7B). When calculated on a protein basis, H-ras contained 43 nmol *S*-

nitrosothiol/mg of protein, as compared with 15 nmol *S*-NO/mg for the total cytosolic protein. This result is consistent with the high concentration of reactive cysteines in H-ras (four reactive cysteines per mole), a value that is undoubtedly higher than the average soluble protein in NIH-3T3 cells. Thus, H-ras may be a major target for protein *S*-nitrosylation reactions.

The data on *S*-glutathiolation of H-ras from Table 1 (determined from protein-bound radioactivity) shows that *S*-nitrosocysteine generated an average of 8 nmol of GSH/mg H-ras (two determinations), which is equivalent to 0.16 mol per mol. Since the cytosolic protein pool contained an average of 1.6 nmol of GSH/mg (Figure 7B), the number of reactive cysteines available for *S*-glutathiolation on H-ras is also greater than the average.

DISCUSSION

Reactivity of cysteines in H-ras

Of the six cysteine residues on H-ras, four (118, 181, 184 and 186) are surface exposed as shown by structural, chemical and mutational studies [10,11,27]. Although Cys-181, Cys-184 and Cys-186 are known to be modified by attached lipid molecules in intact cells, all of the reactive cysteines are potentially oxidized by oxidative or nitrosative processes during normal and pathological cellular events. The premise for this study was that reactive sulphhydryls may be modified by oxidative reactions that lead to *S*-nitrosylated forms, *S*-thiolated forms, or even irreversibly oxidized acidic forms such as the sulphinic or sulphonic acid. This study demonstrates that the protein chemistry of each of the four cysteines may lead to reagent-specific modification reactions. Figure 8 summarizes the oxidized forms of H-ras that were observed in this study. *S*-nitrosylated forms of each reactive cysteine were observed, while negatively charged IAA reacted only with Cys-181, Cys-184 and Cys-186. A second negatively charged adduct, i.e. glutathione, was found to react with only two sites on the C-terminal tail of the protein. Neither of the reagents was able to react readily with Cys-118. The negatively charged protein surface around Cys-118 may account for the lack of reactivity with negatively charged chemicals. In keeping with this hypothesis, it was observed that cystamine, a positively charged reagent, reacted readily with Cys-118 and a second site that may be located in the C-terminal tail region of the molecule.

It has been suggested that *S*-nitrosylation of Cys-118 in H-ras occurs readily in Jurkat cells treated with NO [9]. It was proposed that this modification accounted for the increase in GTP binding observed. The chemistry of Cys-118 described in the present paper is in keeping with facile *S*-nitrosylation of Cys-118 *in vivo*

Figure 8 Model of the modification reactions observed in this study

and it supports the possibility that this modification may be an important modified form of H-ras during nitrosative stress.

Oxidative and nitrosative modification of H-ras in intact cells

Since the role of H-ras oxidation in signalling has been described previously [4–9,17], it was important to determine the nature of oxidative modifications potentially mediated by oxidative agents that might occur in intact cells. For these experiments diamide was chosen as a model cellular stimulant, since this simple oxidant penetrates cell membranes and its unique mechanism of action results in the selective formation of both low-molecular-mass and protein mixed disulphides *in vivo* [32,33]. The disulphides formed are easily reduced by cellular metabolism so that effects generated by diamide are transient, the length of observable effects being dose-dependent. Figure 6 summarizes this study. Because the elimination of either Cys-118 or Cys-181/Cys-184 did not prevent *S*-glutathiolation, we can conclude that either Cys-186 is the cysteine which is predominantly modified, or that modification was distributed evenly among several sites.

S-nitrosocysteine was chosen as the model for *S*-nitrosylation of H-ras in cells. The predominant protein sulphydryl modification using this reagent was *S*-nitrosylation (Figure 7). Lesser, but still significant, amounts of *S*-thiolation (both *S*-glutathiolation and *S*-cysteinylation) occurred as a result of *S*-nitrosocysteine treatment of cells (Figure 7). H-ras appears to conform to this model, with *S*-nitrosylation being the dominant modification.

Role of *S*-nitrosocysteine in lipid turnover

Three of the cysteines at the C-terminus of H-ras are lipidated, which means that any of the above oxidative events are in direct competition with lipidation events of H-ras in cells. However, two of these cysteines are palmitoylated, and constant turnover of the palmitate on cysteine residues suggests that palmitoylation can be affected by oxidative/nitrosylating conditions in cells [17,18]. Recent work shows that *S*-nitrosocysteine caused an increase in turnover of the palmitates at Cys-181 and Cys-184 [17]. Accelerated palmitate turnover was seen using extracellular *S*-nitrosocysteine concentrations as low as 1 mM, but was most pronounced at 4 mM *S*-nitrosocysteine [17]. Figure 7 shows that both the protein thiols and the low-molecular-mass thiol pool are significantly *S*-nitrosylated under similar conditions. Cys-181 and Cys-184 are quite likely to be modified in this regime. Modification of 1 cysteine per molecule of H-ras (Figures 6 and 8), coupled with the lack of specificity of *S*-nitrosylation/*S*-thiolation of H-ras shown in this paper, suggest that *S*-nitrosylation or *S*-thiolation of H-ras could inhibit palmitoylation by limiting the availability of the reactive protein sulphydryl sites for modification. Maximal modification of proteins occurs within 3 min of *S*-nitrosocysteine addition and decreases thereafter (Figure 7). However, protein modification remains substantial for at least 30 min [19], which means that a causal relationship between H-ras *S*-nitrosylation and H-ras palmitate turnover cannot be ruled out.

Acyl protein thioesterase (APT1) has been shown actively to remove palmitate from G α and H-ras [36] and appears to depalmitoylate endothelial nitric oxide synthase (eNOS) in a Ca²⁺-calmodulin dependent fashion [37]. It is therefore possible that the regulation of this protein could be responsible for the effect of *S*-nitrosocysteine on palmitate turnover in H-ras. The role of this protein and its possible oxidative regulation are still to be elucidated.

Alternatively, an increase in free cysteine resulting from an influx of *S*-nitrosocysteine may be responsible for alterations in the steady-state palmitoylation of H-ras by an increase in chemical deacylation of H-ras. It is known that the amount of free thiols in cells treated with *S*-nitrosocysteine is much different than in untreated cells (Figure 7A). In particular, the pool of reduced cysteine increases 10-fold within 3 min of addition of *S*-nitrosocysteine, while at the same time GSH concentration decreases to approximately one-half of pretreatment levels (Figure 7A). At 10 min after *S*-nitrosocysteine treatment, cysteine levels are still four times their pretreatment values, while GSH is still significantly depressed (Figure 7A). Previous work in NIH-3T3 cells showed similar cysteine and GSH dynamics with *S*-nitrosocysteine treatment, and additionally, that levels of cysteine and GSH were perturbed for at least 30 min after addition of *S*-nitrosocysteine [19]. The disruption of steady-state dynamics of palmitate turnover may thus be caused by the change in composition of the free thiol pool, resulting in the rapid cleavage of the thioester by the more reactive cysteine pool via transacylation reactions [38]. Thus, the disruption in the composition of the free thiol pool may disrupt palmitate turnover at the level of protein thiol availability. Since *S*-nitrosocysteine caused no decrease in the amount of membrane-bound H-ras [17], reacylation may be rapid due to the location of H-ras at membranes containing palmitoyl-acyltransferase activity [39,40]. Substrate (i.e. palmitoyl-CoA) availability may be similarly affected by changes in the composition of the free thiol pool [38]. Significantly, in primary hepatocytes, cysteine levels rose 10-fold initially after *S*-nitrosocysteine addition, but GSH levels were unchanged [19]. The differences in thiol pools, especially GSH levels, between hepatocytes and NIH-3T3 cells provides a potential mechanism for observed differences between cell lines in ERK activation in response to oxidants [17].

Role of H-ras in oxidative perturbation of signal transduction

Since it has been suggested that the concentration of *S*-nitrosothiols in plasma is approx. 7 μ M [41] in normal humans, protein *S*-nitrosylation may not be extensive in the absence of a nitrosative stimulus [19]. However, activated neutrophils or macrophages generate considerably enhanced concentrations of both oxidant and nitrosylating materials, and these may affect both cells generating these oxidants and cells in the vicinity of this burst of oxidants [28,42,43]. If the effect of 1 mM *S*-nitrosocysteine (Figure 8) is compared with expected biological events, one might expect that 10% of the H-ras would be oxidatively modified by a 10-fold increase in the normal plasma concentration of nitrosothiols. If *S*-nitrosothiols actually increased to millimolar concentrations, 100% of H-ras might be oxidized.

Previously it was shown that 4 mM *S*-nitrosocysteine inhibited GTP binding and ERK activation, as measured by serum-induced phosphorylation of ERK1 and ERK2 in NIH-3T3 cells [17]. On the other hand, reports also suggested that ERK1 and ERK2 were activated by exposure of other cell types to other oxidants [4–9]. Thus apparently the H-ras pathway may be either activated or inactivated, possibly in a cell type-specific manner, by oxidants of different types of amounts. It is possible that the complex array of reactive sulphydryls on H-ras provides an elegant mechanism for this dual regulatory effect despite the lack of specificity for modification of any particular sulphydryl. In order to activate H-ras-mediated events, only a small fraction of the protein need be oxidized on Cys-118, since small increases in GTP binding can lead to significant activation of the ERK pathway [9]. Since this site is resistant to *S*-glutathiolation,

stimulatory events might be initiated by nitrosothiols, resulting in *S*-nitrosylated H-ras. Inactivation of H-ras-mediated events might require either a larger nitrosative stimulus or one that resulted in uniquely *S*-glutathiolated species of H-ras, thus producing modifications in sites involved in lipid modification of the protein. High oxidant levels [19] may also inactivate other proteins, leading to massive metabolic alterations. Although the experiments reported here suggest a multiple array of sulphhydryl-mediated effects on the function of H-ras, more detailed studies of both *S*-nitrosylation and *S*-glutathiolation should provide a basis for understanding much of what will turn out to be an important series of oxidative regulatory events.

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