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S-glutathionylation regulates GTP-binding of Rac2

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ABSTRACT

Phagocyte NADPH oxidase catalyzes the reduction of molecular oxygen to superoxide and is essential for defense against microbes. Rac2 is a low molecular weight GTP-binding protein that has been implicated in the regulation of phagocyte NADPH oxidase. Here we report that Cys^{157} of Rac2 is a target of S-glutathionylation and that this modification is reversed by dithiothreitol as well as enzymatically by thioltransferase in the presence of GSH. S-glutathionylated Rac2 enhanced the binding of GTP, presumably due to structural alterations. These results elucidate the redox regulation of cysteine in Rac2 and a possible mechanism for regulating NADPH oxidase activation.

1. Introduction

The NADPH oxidase of phagocytes, an important element of host defense against microbial infection, catalyzes the reduction of oxygen to superoxide anion (O_2^-) using NADPH as the electron donor [1]. Rac2, a small G-protein that forms one of the components of the NADPH oxidase enzyme system, has been shown to be crucial for NADPH oxidase activity [2]. In resting cells, Rac2 remains GDP-bound in the cytoplasm. Once activated, Rac2 becomes GTP-bound and moves to the NADPH oxidase enzyme complex in the plasma membrane [3]. The cytosolic oxidase subunit $p67^{phox}$ is a target of Rac2 and contains an activation domain that regulates flavocytochrome b_{558} comprised of two integral membrane proteins, $gp91^{phox}$ and $p22^{phox}$ [4].

GSH, a major low molecular weight antioxidant is present in mammalian cells at millimolar concentrations. Additionally, the ratio of GSH to GSSG, the oxidized form of GSH, is critical for the cellular redox balance [5]. Protein S-glutathionylation is a post-translational modification of protein sulfhydryl groups under moderate oxidative stress, in which a reversible mixed disulfide bond is formed between a protein thiol and GSH [6]. Many previous reports have demonstrated S-glutathionylation (GSS-) in isolated or purified proteins or in cells or tissues exposed to nonspecific thiol oxidants such as diamide or H_2O_2. [5,7]. A growing list of enzymes, including carbonic anhydrase III [6], tyrosine hydroxylase [7], creatine kinase [8], cAMP-dependent protein kinase [9], HIV-1 protease [10], glyceraldehyde-3-phosphate dehydrogenase [11], protein kinase C [12], guanylate cyclase [13] and mitochondrial $NADP^+$-dependent isocitrate dehydrogenase [14], and transcription factors, such as c-Jun, NFκB and IκB [15–18], are potentially influenced by the formation of protein adducts with glutathione.

In the present study, we report that Rac2 was modified by the formation of a mixed disulfide at Cys^{157}, and that this modification was reversed by dithiothreitol (DTT) and by cytosolic thioltransferase (glutaredoxin1, Grx1), a thiol-disulfide oxidoreductase. In addition, we observed structural alterations and augmentation of GTP binding to the S-glutathionylated Rac2. These results help elucidate a modification to the redox regulation of cysteine in Rac2 and a possible mechanism underlying the regulation of NADPH oxidase activation.

2. Materials and methods

2.1. Materials

GSH, GSSG, *N*-acetylcysteine (NAC), DTT, *N*-ethylmaleimide (NEM), *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP), GSH-agarose and 8-anilino-1-naphthalene sulfonic acid (ANSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Mant-GTP was from Molecular Probes (Eugene, OR). Anti-GSH antibody was purchased from ViroGen (Watertown, MA) and streptavidin-horseradish peroxidase (HRP) was purchased from Cell Signaling (Beverly, MA).

2.2. Site-directed mutagenesis and preparation of recombinant proteins

Site-directed mutagenesis was performed using a Quick-change™ site-directed mutagenesis kit (Stratagene). In order to prepare recombinant Rac2 and mutant Rac2 proteins, *Escherichia coli* were transformed with pGEX-4T containing the cDNA insert encoding Rac2 (a kind gift of Dr. Jamel El Benna, INSERM Paris,

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France) and mutant Rac2 constructs were grown and lysed, GST-tagged proteins were purified by affinity chromatography on GSH-agarose. The GST tag then removed from Rac2 using thrombin as previously described [19]. Protein concentrations were measured using a Bio-Rad assay kit.

2.3. In vitro glutathionylation

Recombinant Rac2 (2 μg) in 40 mM Tris–HCl (pH 8.0) was incubated with various concentrations of GSSG at 37 °C for the indicated times. Glutathionylated samples were subjected to SDS–PAGE and immunoblotting with an anti-GSH IgG. *In vitro* experiments with purified Rac2 were also performed with biotin-labeled GSSG. Biotin-GSSG was synthesized by coupling biotin to the primary amino acids of GSSG using EZ-Link™ Sulfo-NHS-Biotin (Thermo) as the biotinylating reagent as previously described [20]. Biotin labeling was identified with streptavidin-HRP.

2.4. Immunoblot analysis

The proteins were separated on a 10% SDS–polyacrylamide gel, transferred to nitrocellulose membranes, and subsequently, subjected to immunoblotting with anti-GSH IgG. Primary antibody binding was then detected with HRP-labeled anti-rabbit IgG and an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech). To detect glutathionylatin Rac2 was mixed with a 5× SDS sample buffer without reducing reagents and supplemented with 5 mM NEM to block unreacted thiol groups. The protein was then subjected to SDS–PAGE followed by electro blotting onto nitrocellulose membranes.

2.5. Structural analysis

Steady-state intrinsic fluorescence measurements were performed using a Shimadzu RF-5301 PC spectrofluorophotometer with the sample compartment maintained at 22 °C. A 150 W xenon source was used. The slit-width was fixed at 5 nm for excitation and emission. Samples were subjected to excitation at 278 nm, and the emission was monitored between 300 and 400 nm. Various forms of Rac2 in 25 mM potassium phosphate buffer, pH 7.0/50 mM KCl were incubated with ANSA (100 μM). The fluorescence emission spectra (370 nm excitation) of the different mixtures were monitored with the spectrofluorometer. ANSA binding to the protein was identified by subtracting the emission spectrum of ANSA from that of ANSA in the presence of the protein.

2.6. Analysis of mant-GTP binding by Rac2

Mant-GTP (100 nM) and Rac2 (100 nM) were added to assay buffer (20 mM Tris–HCl, 3 mM NaCl, 50 mM KCl, and 2.5 mM $MgCl_2$) in a cuvette at a final volume 2 ml. The samples were then analyzed with a spectroflurometer at room temperature at an excitation wavelength of 355 nm (10-nm bandwidth) and the emission spectra from 370 to 600 nm (5-nm bandwidth) were recorded. The baseline spectrum of the buffer alone was subtracted from all spectra measured.

2.7. Replicates

Unless otherwise indicated, each result presented in the paper is representative of at least three separate experiments.

3. Results and discussion

Regulation of biological activity by the reversible modification of protein thiol, like phosphorylation, has been suggested to be an important mechanism of turning on and off proteins particularly in response to oxidative stress [5]. Proteins containing cysteine residues are susceptible to protein S-glutathionylation, the reversible covalent addition of glutathione to sulfhydryl groups on target proteins [5]. In this study, we present evidence indicating that Rac2 can be modified by reversible S-glutathionylation.

It has been shown that a polyclonal anti-GSH antibody is very useful for detecting S-glutathionylation [16]. When Rac2 was incubated with various concentrations of GSSG and subjected to non-reducing Western Blot analysis with a polyclonal anti-GSH antibody, the intensity of the immunoreactive band was increased in a concentration- and time-dependent manner (Fig. 1A and B). *N,N′*-Biotinyl GSSG is proved to be a useful tool for studying protein S-glutathionylation [20]. Thus, recombinant Rac2 was treated with biotin-GSSG in the present study as a complementary approach. Fig. 1C shows non-reducing Western Blots probed with streptavidin-HRP to detect Rac2 undergoing S-glutathionylation following this treatment. The main feature of S-glutathionylation that indicates this process may be a possible regulatory mechanism is its

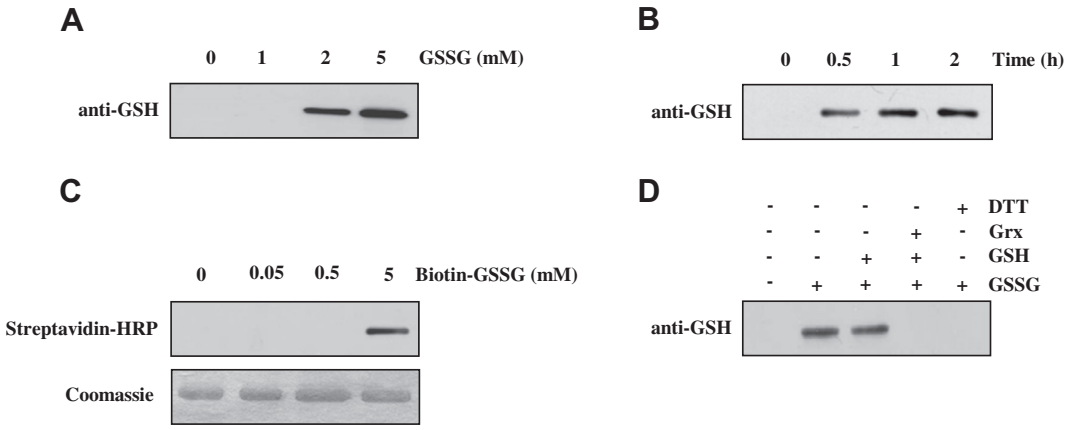

Fig. 1. Glutathionylation of Rac2. (A) Immunochemical analysis of GSS-Rac2. After incubating Rac2 protein with various concentrations of GSSG for 30 min at 37 °C, the samples were subjected to SDS–PAGE for immunoblotting with anti-GSH IgG. (B) After incubating Rac2 with 5 mM GSSG for various lengths of time at 37 °C, the samples were subjected to SDS–PAGE and immunoblotting with anti-GSH IgG. (C) Recombinant Rac2 was incubated with various concentrations of biotin-GSSG for 1 h. The proteins were then subjected to Western Blotting, probed with streptavidin-HRP and antibody binding was detected with the ECL reagent. (D) Reversal of glutathionylated Rac2 by thiols. Rac2 was incubated with 5 mM GSSG for 30 min and subsequently treated with 10 mM DTT, 0.5 mM GSH or 5 μg Grx1 in the presence of 0.5 mM GSH for 30 min at 37 °C. Samples were subjected to SDS–PAGE followed by immunoblotting with anti-GSH IgG.

reversibility [5]. As shown in Fig. 1D, the addition of 10 mM DTT reversed S-glutathionylation, suggesting that GSSG modifies susceptible cysteine(s) on the protein through the formation of protein-SSG species. Grx, or thioltransferase, is known to specifically reverse protein-glutathione mixed disulfides by utilizing GSH as an electron donor [21]. Although 0.5 mM GSH alone was not effective, 3.3 μM Grx1 and 0.5 mM GSH were able to deglutathionylate Rac2-SSG.

In order to determine whether S-glutathionylated cystein(s) in Rac2 are susceptible to sulfhydryl modifying agents, Rac2 was allowed to simultaneously react with 5 mM GSSG and various concentrations of NEM, NAC, and SNAP for 1 h. As shown in Fig. 2 A, a dose-dependent decrease of S-glutathionylated Rac2 was subsequently observed. To further confirm which cysteine residue was a target for S-glutathionylation, seven single C → A mutant Rac2 proteins (C6A, C18A, C81A, C105A, C157A, C178A, and C189A) were prepared. When mutant proteins were exposed to 5 mM GSSG, only the C157A mutant was not glutathionylated among the seven mutant Rac2 proteins, confirming that Cys^{157} is a target of Rac2 S-glutathionylation (Fig. 2B).

Modification of protein thiols can potentially affect protein conformation. Indeed, the modulation of intrinsic tryptophan fluorescence and the binding of ANSA to S-glutathionylated Rac2 indicate structural alterations. Upon excitation of native Rac2 at 278 nm, a fluorescence emission spectrum typical for tryptophan residues with a maximum at 337 nm was observed. The fluorescence spectra of native and S-glutathionylated Rac2, normalized to the protein content, showed that modified Rac2 displayed a dose-dependent decrease in quantum yield of the emission spectra and a red shift of the maximum emission wavelength (Fig. 3A). To detect increases in protein flexibility during the partial unfolding of S-glutathionylated Rac2, binding of the fluorescent probe ANSA was used to detect the accessibility of Rac2 hydrophobic regions. Binding can be easily monitored since it is accompanied by an increase in fluorescence associated with the transfer of the ANSA from a hydrophilic to a hydrophobic environment [22]. Rac2 exposed to various concentrations of GSSG for 1 h, it bound to the hydrophobic probe ANSA more efficiently than does the native protein (Fig. 3B).

Previous reports have shown that the binding of methylanthraniloyl guanosine-5′-[β,γ-imido] triphosphate (mant-GppNHp) or mant-GTP to Rac results in an increased fluorescence at 440–445 nm, the emission peak of the mant moiety [23,24]. We therefore utilized mant-GTP as a reporter group to investigate the effect

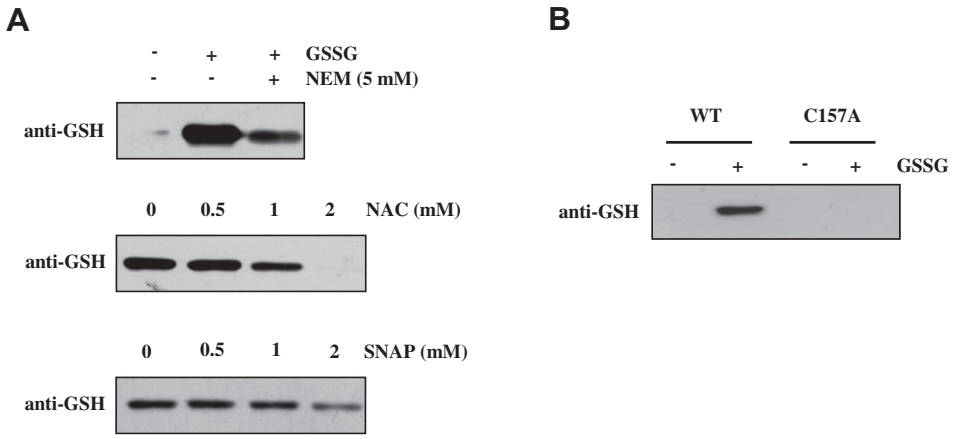

Fig. 2. Effect of sulfhydryl modifying reagents on Rac2 glutathionylation (A) After incubating Rac2 with 5 mM GSSG in the presence or absence of various concentrations of NAC, NEM or SNAP for 1 h at 37 °C, the proteins were separated by SDS–PAGE and immunoblotted with anti-GSH IgG. (B) Identification of GSS-Cys in Rac2 with a site-directed mutagenesis. After incubation with 5 mM GSSG for 1 h at 37 °C, wild-type and the Rac2C157A mutant were subjected to SDS–PAGE followed by immunoblotting with anti-GSH IgG.

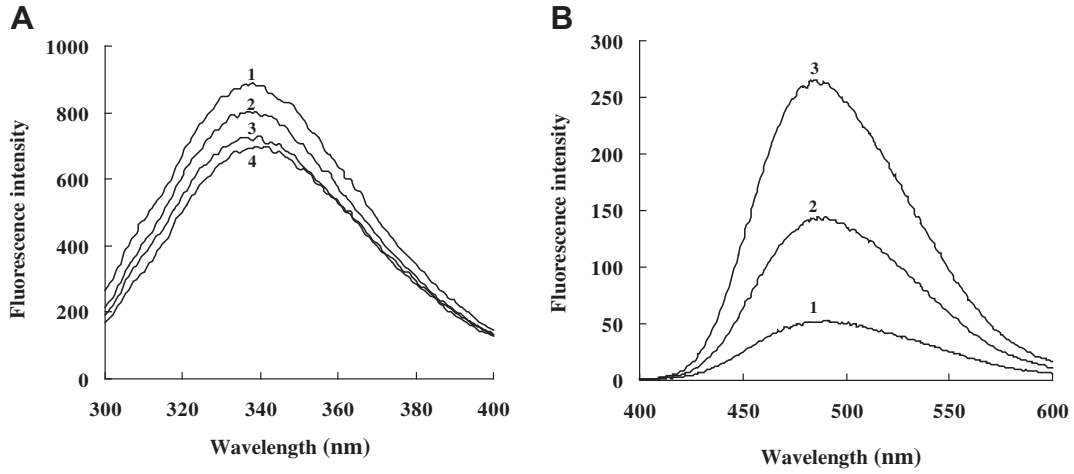

Fig. 3. Changes in Rac2 structure following glutathionylation. (A) Steady-state emission spectra of intrinsic fluorescence of native Rac2 (*line 1*) and Rac2 treated with 2, 5 and 10 mM GSSG for 1 h at 37 °C (*lines 2–4*, respectively) were analyzed with a spectrofluorimeter. Spectra were obtained using an excitation wavelength of 278 nm and excitation and emission slits of 5 nm. (B) Spectrofluorometric analysis of ANSA binding to the glutathionylated Rac2. Emission spectra from 400 to 600 nm (370 nm excitation) of ANSA (100 μM) bound to native Rac2 (*line 1*) and Rac2 treated with 5 and 10 mM GSSG for 1 h at 37 °C (*lines 2* and *3*, respectively) were obtained.

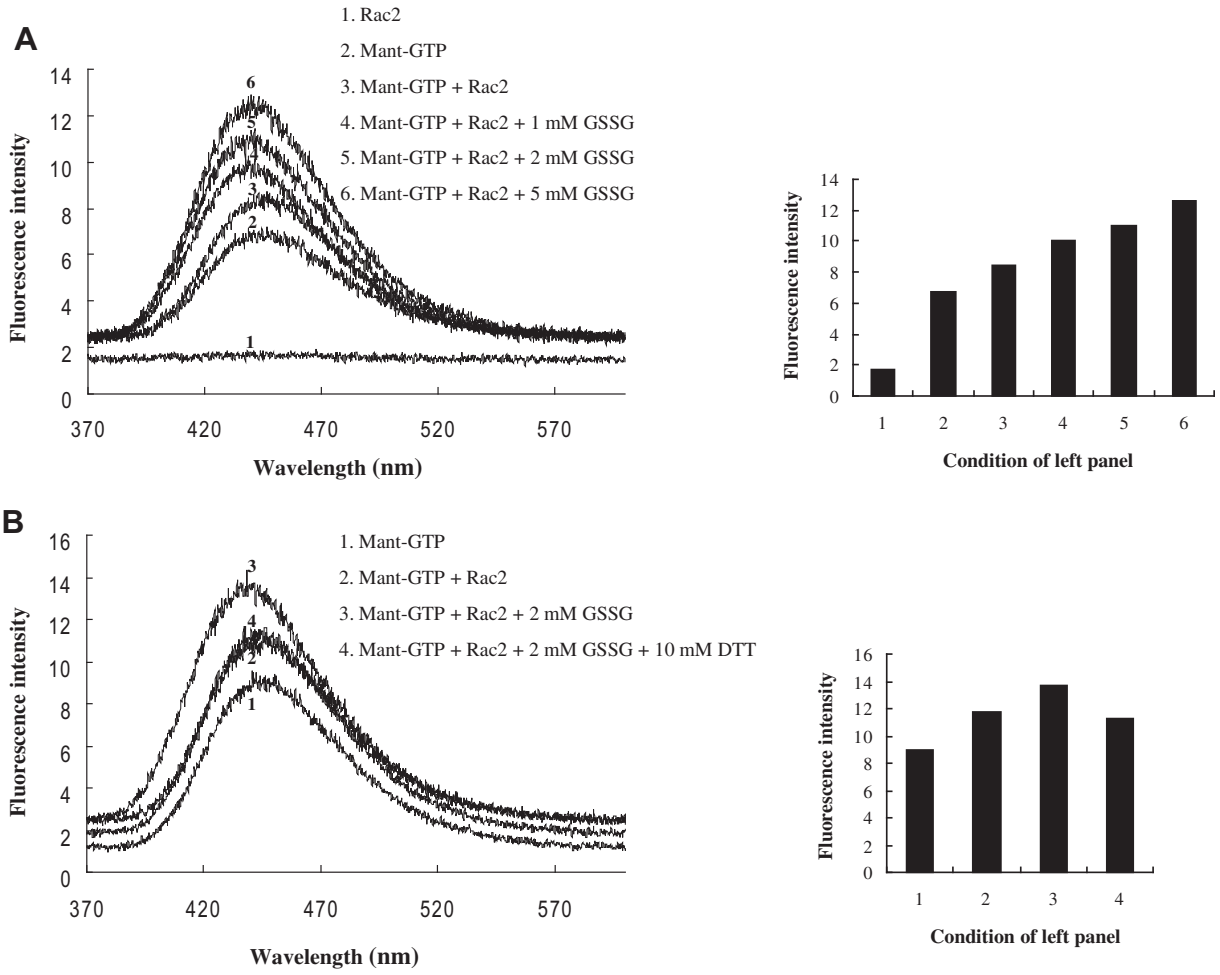

Fig. 4. Glutathionylation of Rac2 enhances Rac2 GTP-binding activity. (A) Rac2 (0.1 μM) was incubated with buffer or 1, 2, and 5 mM GSSG for 1 h at 37 °C. Following pre-incubation with 100 nM mant-GTP was added, and fluorescence spectra of the sample were collected as described in material and methods. Peak fluorescence at 440 nm was determined from the spectra. (B) The effect of 10 mM DTT on the glutathionylation-induced augmentation of mant-GTP fluorescence in Rac2. Peak fluorescence at 440 nm was determined from the spectra.

of Rac2 S-glutathionylation on GTP binding. As shown in Fig. 4A, preincubation of Rac2 with various concentrations of GSSG increased the mant fluorescence in a concentration-dependent manner, suggesting that S-glutathionylation of Rac2 enhanced the subsequent binding of mant-GTP. Furthermore, the addition of 10 mM DTT effectively blocked S-glutathionylation-induced augmentation of mant-GTP fluorescence in Rac2 (Fig. 4B).

Recently, it was demonstrated that increased levels of oxidants in both endothelial and smooth muscle cells can directly activate Ras G protein via S-glutathionylation of a reactive thiol on Cys^{118} and triggers downstream signaling of the phosphorylation of ERK and AKT [25,26]. These studies showed that modification of Cys^{118} in Ras directly promotes guanine nucleotide binding affinity, thus changing the activity of this factor. A mechanistic understanding of how S-glutathionylated Cys^{157} in Rac2 leads to enhanced guanine nucleotide binding will be likely provided by solving the X-ray crystal structure. In addition, the effect of Rac2 post-translational modifications on the *in vivo* regulation of NADPH oxidase activity warrants further investigation.

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