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Original Contribution

Glutathiolated Ras: Characterization and implications for Ras activation

G. Aaron Hobbs [a], Marcelo G. Bonini [b], Harsha P. Gunawardena [a,c], Xian Chen [a,c], Sharon L. Campbell [a,d,*]

[a] Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC 27599, USA
[b] Section of Cardiology and Department of Pharmacology, College of Medicine, University of Illinois, Chicago, IL 60612, USA
[c] Program in Molecular Biology and Biotechnology, University of North Carolina, Chapel Hill, NC 27599, USA
[d] Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599, USA

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ABSTRACT

Ras GTPases cycle between active GTP-bound and inactive GDP-bound forms to regulate a multitude of cellular processes, including cell growth, differentiation, and apoptosis. The activation state of Ras is regulated by protein modulatory agents that accelerate the slow intrinsic rates of GDP dissociation and GTP hydrolysis. Similar to the action of guanine-nucleotide exchange factors, the rate of GDP dissociation can be greatly enhanced by the reaction of Ras with small-molecule redox agents, such as nitrogen dioxide, which can promote Ras activation. Nitrogen dioxide is an autoxidation product of nitric oxide and can react with an accessible cysteine of Ras to cause oxidation of the bound guanine nucleotide to facilitate Ras guanine nucleotide dissociation. Glutathione has also been reported to modify Ras and alter its activity. To elucidate the mechanism by which glutathione alters Ras guanine nucleotide binding properties, we performed NMR, top-down and bottom-up mass spectrometry, and biochemical analyses of glutathiolated Ras. We determined that treatment of H-Ras, lacking the nonconserved hypervariable region, with oxidized glutathione results in glutathiolation specifically at cysteine 118. However, glutathiolation does not alter Ras structure or biochemical properties. Rather, changes in guanine nucleotide binding properties and Ras activity occur upon exposure of Ras to free radicals, presumably through the generation of a cysteine 118 thiyl radical. Interestingly, Ras glutathiolation protects Ras from further free radical-mediated activation events. Therefore, glutathiolation does not affect Ras activity unless Ras is modified by glutathione through a radical-mediated mechanism.

Ras proteins belong to a large superfamily of GTPases that bind to GDP and GTP with high specificity and affinity. They function as molecular switches that cycle between the inactive GDP-bound and the active GTP-bound forms. Once activated, Ras GTPases interact with a variety of effectors to activate signaling pathways that regulate gene expression, cell growth and differentiation, and programmed cell death [1–3]. The structural differences between the GDP- and the GTP-bound states of Ras are primarily localized within two regions, Switch I (residues 30–37) and Switch II (residues 60–76) [4]. The intrinsic rates of GDP exchange and GTP hydrolysis are too slow to respond to cell signaling events [5], and consequently, protein factors associate with Ras and accelerate these rates in a regulated manner. Guanine nucleotide exchange factors (GEFs)[1] accelerate the exchange of bound GDP for GTP, which leads to Ras activation [6], whereas GTPase activating proteins (GAPs) inactivate the GTPase by facilitating GTP hydrolysis [7,8]. Ras is considered the most prevalent oncogene in human cancer, and oncogenic mutations have been identified in approximately 30% of human tumors [9,10]. Oncogenic Ras mutations render Ras constitutively active by impeding down-regulation by GAPs [11].

In addition to GEFs, reactive oxygen and nitrogen species (ROS, RNS) have been shown to enhance guanine nucleotide exchange and activate Ras [12]. Two of the most common oxidative modifications identified for Ras in vivo are nitrosation and glutathiolation [13]. Specifically, nitrogen dioxide ($^{\bullet}NO_2$) has been shown to induce Ras guanine nucleotide dissociation by causing guanine base oxidation through reaction with cysteine

Abbreviations: BAEC, bovine aortic endothelial cell; DAF, diaminofluorescein; DEANO, diethylammonium (*Z*)-1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate; eNOS, endothelial nitric oxide synthase; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factors; HCD, higher energy collisional dissociation; HSQC, heteronuclear single quantum coherence spectroscopy; MANT-GDP, 2′,3′-*O*-(*N*′-methylanthraniloyl)guanosine 5′-*O*-diphosphate; MS, mass spectrometry; $^{\bullet}NO_2$, nitrogen dioxide; Ras^{SNO}, S-nitrosation of Ras; Ras^{SSG}, glutathiolated Ras; ROS, RNS, reactive oxygen and nitrogen species; SOS, son of sevenless; SIM, selected ion monitoring; VSMC, vascular smooth muscle cell.

* Corresponding author at: University of North Carolina, Department of Biochemistry and Biophysics, Chapel Hill, North Carolina, 27599, United States. Fax: +(919) 966 2852.
E-mail address: campbesl@med.unc.edu (S.L. Campbell).

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118 (Cys^{118}) [14]. As the GTP:GDP ratio in cells is approximately 10:1, oxidation-mediated GDP dissociation can promote GTP loading of Ras, analogous to the action of GEFs [15]. Although the reactions of nitric oxide and its autoxidation product, $^{\bullet}NO_2$, with Ras have been characterized, it is less clear how other thiol modifications (i.e., glutathiolation) affect Ras activity. As numerous studies have described activity modulation of Ras in the presence of ROS and RNS, we refer the reader to two recent reviews that detail the redox regulation of Ras and Ras-related GTPases [12,16].

Ras contains a solvent accessible cysteine (X) in the nucleotide-binding NKXD motif. Although this cysteine is conserved in H-, K-, and N-Ras, it does not form interactions with other residues in Ras or the guanine nucleotide ligand and is poorly conserved in the Ras superfamily. We have previously postulated that Cys^{118} is conserved in N-, K-, and H-Ras because of its role in the redox regulation of Ras [15]. In fact, Cys^{118} has been shown to react in vitro and in vivo with a variety of thiol-oxidizing agents, including oxidized glutathione, superoxide, and nitric oxide-derived oxidants [13]. We have previously characterized a redox-inactive variant of Ras (Ras^{C118S}) that retains structural and biochemical properties similar to those of wild-type Ras (Ras^{WT}) [17,18]. Ras^{C118S} has been used in numerous in vitro and cell-based studies to discriminate between direct and indirect mechanisms of Ras activity modulation by Cys^{118}-mediated oxidation [reviewed in [12,13]]. As we have previously shown that S-nitrosation of Ras (Ras^{SNO}) at Cys^{118} does not significantly alter Ras structure, biochemical properties, or binding to the Ras-binding domain of Raf-1 [19], we postulated that NO modulates Ras activity by formation of a thiyl-radical intermediate, which leads to oxidation of the bound guanine base, likely through electron transfer. In support of this premise, we have previously shown that treatment of Ras with $^{\bullet}NO_2$ promotes the oxidation and release of the bound guanine nucleotide [14]. In addition, we have recently detected the presence of a Ras-thiyl radical upon treatment of Ras^{WT} with $^{\bullet}NO_2$ using the immuno-spin trapping reagent 5,5-dimethyl-1-pyrroline *N*-oxide; no radical was detected when Ras^{C118S} was used [20]. Thus, we propose that Ras thiyl-radical formation, as opposed to S-nitrosation, induces oxidation and release of the guanine nucleotide base, which can lead to Ras activation.

In addition to S-nitrosation, several studies have observed that Ras can undergo glutathiolation. This oxidative modification was suggested to increase nucleotide exchange and promote Ras activation [21–27]. In particular, detection of Ras glutathiolation coincident with Ras activation was observed after exposure of bovine aortic endothelial cells (BAECs) to peroxynitrite [21,27], addition of angiotensin II to vascular smooth muscle cells (VSMCs) [26,28], and exposure of H_2O_2 to rat ventricular myocytes [23,25]. However, addition of these agents to cultured cells can produce redox agents capable of protein thiyl radical formation. For example, peroxynitrite can react with cellular CO_2 to produce $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ radicals, and under acidic conditions (peroxynitrite pK_a is $\sim$6.6), peroxynitrite can decompose to hydroxyl radical and $^{\bullet}NO_2$ [29]. Furthermore, peroxynitrite and angiotensin II can dysregulate several kinase pathways, including the PI3K/Akt pathway, and increase the production of cellular NO by activation of endothelial nitric oxide synthase (eNOS) [30,31]. The NO produced by eNOS can autoxidize to produce $^{\bullet}NO_2$, which is a powerful oxidant capable of thiyl radical formation. Consistent with observations of Liaudet et al. [30] and Ushio-Fukai et al. [31], the PI3K/Akt pathway was activated after exposure of BAECs to peroxynitrite [21]. Intriguingly, in a pancreatic cancer cell line and a severe combined immunodeficiency/Beige mouse model, activation of PI3K has been shown to activate Ras via eNOS stimulation through a redox-mediated mechanism dependent on Cys^{118} [32]. In addition, peroxynitrite and angiotensin II signaling both activate NADPH oxidase, which can produce waves of reactive oxygen species long after peroxynitrite decomposition [33]. Furthermore, H_2O_2 can produce free radicals through the Fenton reaction by reacting with transition metals present in cells. Thus, based on our previous characterization of nitrosated Ras [19], we postulate that Ras activation observed by Clavreul et al. [21,27] and Adachi et al. [26] results from a radical-mediated mechanism, which occurs before the modification by glutathione. However, because of the larger size of glutathione compared to NO, it was hypothesized that Ras glutathiolation at Cys^{118} causes structural changes in the nucleotide binding pocket, leading to perturbation of guanine nucleotide binding and an increased rate of nucleotide exchange [21]. As Cys^{118} is critical for the radical-mediated regulation of Ras activity, we sought to circumvent the confounding factors of peroxynitrite and angiotensin II use in cells by directly determining whether glutathiolation at Cys^{118} alters Ras structure and activity in vitro.

Although Ras has been shown to be glutathiolated in cells [21,23,26,27], it is unclear how glutathiolation alters Ras activity. Therefore, we employed nuclear magnetic resonance (NMR) and fluorescence-based biochemical assays to assess whether glutathione modification of Ras perturbs Ras structure or activity. We found that treatment of Ras with oxidized glutathione leads to glutathiolation specifically at Cys^{118}, which does not alter Ras tertiary structure or guanine nucleotide binding. These results are consistent with our previous observations that S-nitrosation of Ras Cys^{118} does not perturb the structure or activity of Ras [19]. Therefore, our data suggest that glutathiolation can affect Ras activity only if modification proceeds through a radical-mediated reaction. Moreover, Ras glutathiolation prevents further redox-mediated activation of Ras by free radical-based mechanisms, which may serve to protect Ras from future radical-mediated oxidation events under conditions of oxidative stress.

Materials and methods

Ras purification and glutathiolation

Truncated human *H-ras* ($H\text{-}Ras^{1-166}$) was cloned into the pQlinkH vector (Addgene, Cambridge, MA, USA), which contains an N-terminal 6 × -His purification tag followed by a tobacco etch virus protease cleavage site for removal of the affinity tag. The hypervariable region of Ras, including the C-terminal CAAX box, was removed as this region does not undergo posttranslational lipid modification in bacteria, it is unstructured, and its removal does not affect guanine nucleotide binding or GTP hydrolysis [34]. All proteins were expressed in BL21(DE3) RIPL cells (Stratagene, La Jolla, CA, USA) and purified following the Qiagen nickel NTA purification protocol (Germantown, MD, USA). Ras was further purified by size-exclusion chromatography (Superdex-75 10/300 GL column; GE Life Sciences, Piscataway, NJ, USA) and judged greater than 95% pure by SDS–PAGE analysis.

Ras was modified with glutathione by the addition of 1000 × oxidized glutathione to purified Ras in glutathiolation buffer (50 mM Tricine, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, and 30 μM GDP) at 37 °C for 15 min. Before the addition of glutathione, Ras was reduced with dithiothreitol for 30 min at pH 8.5 before being buffer-exchanged into glutathiolation buffer that was flushed with N_2 gas to remove dissolved oxygen and prevent autoxidation.

Mass spectrometry of glutathiolated Ras and Ras

Ras mass measurements were performed on an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, San Jose, CA, USA). The mass analysis of intact Ras samples was achieved in full-mass spectrometry (MS), selected-ion monitoring (SIM), and higher