Redox Regulation of Rac1

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ABSTRACT

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(Under the direction of Sharon Campbell)

Rac1 is a ubiquitous 21 kD guanine nucleotide binding protein that is a member of a large superfamily of GTPases, which plays a central role in fundamental cell processes, including cell motility and morphology, gene expression, cell cycle control and survival, as well as cell redox homeostasis. Because Rac1 is involved in so many important processes, deregulated Rac1 activity can yield a number of pathological conditions, such as cancer, cardiovascular disease, and neurological disorders. Mounting evidence suggests that a number of small GTPases, including Rac1, can be regulated by redox agents, and the Campbell lab has demonstrated that a subset of small GTPases have a redox-active Cys proximal to their active site, which can interact with oxidants and alter activation. I demonstrated that the redox-active Cys in Rac1 has a lowered pK_a and is likely to be oxidized at a physiological pH; I selectively oxidized this Cys residue in Rac1 and characterized this modified form of the protein. Further, given recent observations that Rac1 interacts with SOD1 in a redox- and nucleotide-dependent manner, I initiated biochemical and biophysical analyses to quantify and characterize this novel interaction. Replacing the redox active cysteine with an aspartate residue (sulfonated Cys mimetic) abrogated the interaction, which suggests that sulfonic acid oxidation of Rac1 may perturb binding interactions with SOD1. In addition, I identified certain regions in Rac1 that are perturbed upon interaction with SOD1 by NMR.

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5-nitro-GDP, 5-nitro-guanine ribose diphosphate

ALS, amyotrophic lateral sclerosis

DTT, dithiothreitol

eNOS, endothelial NOS

LC-ESI-MS, liquid chromatography electrospray ionization MS

GDI, GDP dissociation inhibitor

GSSG, oxidized glutathione

H_2O_2, hydrogen peroxide

iNOS, inducible NOS

LMW-PTP, low-molecular-weight protein tyrosine phosphatase

MALDI-MS, matrix-assisted laser desorption/ionization MS

MS, mass spectrometry

$NO^{\bullet}$, Nitric oxide

nNOS, neuronal NOS

NOS, nitric oxide synthase

Nox, NADPH oxidase

NIm-DP, 5-guanidino-4-nitroimidazole diphosphate

$O_2^{\bullet -}$, superoxide

$^{\bullet}OH$, hydroxyl radical

$ONOO^-$, peroxynitrite

p-loop, phosphoryl-binding loop

PAK1, p21-activated kinase

PAO, phenylarsine oxide

PI3K, phosphoinositide 3-kinase

RIs, reactive intermediates

RNS, reactive nitrogen species

ROCK, Rho-associated protein kinase

ROS, reactive oxygen species

SOD, superoxide dismutase

VEGF, vascular endothelial growth factor

I. Introduction

Rac1 is a ubiquitous 21 kD guanine nucleotide binding protein that is a member of a large (>150) superfamily of GTPases (*1*). Rac1 plays a central role in fundamental cell processes, including cell motility, morphology, gene expression, cell survival, and cell cycle control (*2*). Found in many organisms, Rac1 is expressed in most cell types and tissues (*3*). The highly related isoforms Rac2 and Rac3 are expressed in only certain cell types; Rac2 is expressed in hematopoietic cells, and Rac3 is expressed in brain cells (*3*). Interestingly, Rac1 is also important for oxidant regulation; it interacts with and activates multiple enzymes responsible for maintaining the redox status of the cell (e.g., NADPH oxidase, "Nox," and nitric oxide synthase, "NOS") (*4, 5*). Moreover, it has recently been shown that Rac1 interacts with SOD1 (superoxide dismutase1) through a nucleotide and redox-dependent mechanism that is abrogated by SOD1 with amyotrophic lateral sclerosis ("ALS")-associated mutations (*6*). Given its involvement in so many important processes, a number of disease states can result from deregulation of Rac1 activity, such as cancer, cardiovascular disease, and neurological disorders (*2, 7-9*).

Mounting evidence suggests that a number of small GTPases, including Rac1, can be regulated by redox agents (*10, 11*). Given the complex chemistry and high reactivity of reactive nitrogen and oxygen species ("RNS" and "ROS"), including hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$), and nitric oxide ($NO^{\bullet}$), it has been difficult to fully discern the specific regulatory mechanisms (*10-12*).

The Campbell lab has demonstrated that a subset of small GTPases (e.g., Rac1, RhoA, and Ras) have a redox-active Cys proximal to their active site, which can interact with radicals (e.g., $O_2^{\bullet-}$ and $NO^{\bullet}$), transfer the radical to bound guanine nucleotide, and alter the activation state of the protein (*13-18*). Certain members of the Rho GTPase subclass have a distinctive redox-active motif (e.g., Rac1 and RhoA) that might be reactive to non-radical and radical oxidants, which have been the subject of previous research efforts (*11*). To study this phenomenon, I developed a method to site-selectively glutathiolate a redox-active Cys in the active site of Rac1 for at an approximately 80% yield. During this process, I noted certain experimental results that indicated an atypically low pK_a at the redox-active Rac1 Cys18 thiol. Pursuant to these observations, I conducted chemical reactivity experiments to measure the pK_a for this cysteine, which is approximately 6.8. This observation indicates that the Cys18 thiol in Rac1 can be selectively glutathiolated, is more reactive (~10-fold) at a physiological pH than other Rac cysteines, and is, thus, prone to oxidative regulation *in vivo*. I also characterized how Rac1 glutathiolation alters its biochemical and biophysical properties *in vitro*. Moreover, I generated and characterized a redox-insensitive Rac1 variant as well as a sulfonic acid mimetic at the Rac1 redox-active residue. Generation of a redox-insensitive variant with similar biochemical properties to Rac1 is a key control experiment that supports a direct relationship between Cys18 oxidation and alterations in the biochemical and biophysical properties of Rac1. Moreover, the redox-insensitive variant has been an invaluable control in cell-based experiments to demonstrate that our observations on the Rac1 oxidation phenomenon are directly mediated through Rac1 Cys18.

Finally, given recent observations that Rac1 interacts with SOD1 in a redox- and nucleotide-dependent manner, I initiated biochemical, NMR, and biophysical analyses to quantify and characterize this novel interaction (*5*). Replacing the redox active cysteine with an aspartate residue abrogated the interaction, which suggests that sulfonic acid oxidation of Rac1 may perturb binding interactions with SOD1. In addition, I identified certain regions in Rac1 that are perturbed upon interaction with SOD1 by NMR.

Herein, I first discuss the relevant background for Rac1, redox-regulation, and the SOD1/Rac1 interaction. Next, I describe and discuss the experiments and observations for redox-regulation of Rac1 as well as the Rac1/SOD1 interaction. Finally, I draw conclusions for these studies and propose future directions for this research.

A. Rac1 and small GTPases

1. The small GTPase activation paradigm

A common characteristic of the small Ras superfamily GTPases and their most predominant feature is the nucleotide switch activation mechanism (Figure 1.1) (*19*). Ras GTPases bind the guanine nucleotides GDP and GTP with picomolar affinity (*19, 20*). Through the nucleotide switch mechanism, the GTPases change conformation depending on whether bound to GDP or GTP (*1, 20*). GDP-bound GTPases are predominantly in an inactive conformation, whereas GTP-bound GTPases adopt an activated form (*1, 20*). The nucleotide-mediated conformational change involves modulation of the conserved switch I and II regions (residues 26-36 and 59-76, respectively; Rac1 numbering) (*1, 21, 22*). The switch regions contain residues that interact with the γ-phosphate, which draws these regions closer to the nucleotide when GTP is bound (*23*). This stabilizes the 'active' GTPase conformation, which is recognized by downstream effectors. When GDP

is bound to the protein, the switch regions form fewer interactions with the nucleotide; thus, switch I and II are more dynamic, sample multiple conformational states, and move further away from the nucleotide (*23*). Downstream effectors bind activated GTPases with significantly higher affinity than the GDP-bound form, and typically, this binding event facilitates a signaling pathway that produces a cell response to a particular stimulus (*24*). Such signaling pathways are usually tightly regulated both spatially and temporally (*25, 26*).

One way that these signaling paths are regulated is through the activation status of GTPases. The dominant paradigm for Ras superfamily GTPases is that activity is regulated by protein modulatory factors. In the cell, small GTPases are regulated by GAPs (GTPases activating proteins) and GEFs (guanine nucleotide exchange factors) (*1*). GAPs downregulate GTPases by catalyzing GTP hydrolysis, which inactivates GTPases (*20, 23*). GEFs facilitate nucleotide exchange; as the GTP concentration is typically at least 10-fold greater than the GDP concentration in the cell, nucleotide dissociation usually activates GTPases *in vivo* (*20, 27*). In addition to GEF-mediated exchange, the Campbell lab has previously demonstrated that radical oxidants (e.g., NO and superoxide) can facilitate nucleotide dissociation in select Ras superfamily GTPases (*13, 15-17*), and I demonstrate that non-radical oxidative modification (e.g., thiol glutathiolation and sulfonation) of Rac1 can also dramatically enhance GDP dissociation. Thus, likely oxidation is an additional mechanism for regulating Rac1 activation.

Examples of GAPs that catalyze Rac1 GTP hydrolysis include p50RhoGAP and n-chimaerin, and GEFs that facilitate nucleotide dissociation in Rac1 include Vav2 and Tiam1 (*27, 28*). For certain proteins in the Rho subclass (e.g., Rac1), GDIs (guanine

nucleotide dissociation inhibitors) are an additional layer of regulation (*29, 30*). GDIs extract Rho subclass GTPases from the membrane and sequester the GDP-bound form through C-terminal prenyl groups, which maintains a pool of inactive GTPases and regulates localization (*30-32*). RhoGDIα and RhoGDIβ have been shown to act on Rac1 (*29, 30*).

However, recent studies have demonstrated that GEFs, GAPs, and GDIs are only a part of the increasingly complex mechanism that regulates small GTPase activity and effector interactions; post-translational modifications are an additional layer of regulation for small GTPases. Most small GTPases require lipid modifications (farnesyl and geranyl-geranyl groups) at the C-terminal portion of the protein ("CAAX box") for membrane insertion, which is typically a requisite step for GEF-mediated localization and activation (*32*). Phosphorylation of Rac1 (Ser71 through Akt phosphorylation) has also been reported to alter interactions with certain effectors (PAK and WASP but not IQGAP, MRCKα, or NF-κB); while it reduces GTP binding, the reduction is only 2-fold, and no information is available on whether GEF interactions are effected (*33-35*). Moreover, SUMO has been shown to modify Rac1-GTP at the polybasic tail (Lys residues 183, 184, 186, and 188) and facilitate "optimal lamellipodia-ruffle formation, cell migration and invasion" (*36*). Finally, ubiquitin/proteasome-mediated Rac1-GTP degradation through polyubiqutination at Lys147 also plays a role in regulating the pool of activated Rac1 (*37, 38*). This same report mentioned that, in their in vitro system, mono-ubiquitinated Rac1 at Lys147 still bound the membrane and could be activated (*37, 38*). They hypothesized that mono-ubiquitinated Rac1 may also have a distinct cell role, but did not offer additional evidence to support this supposition (*37, 38*). I describe in

vitro and cell-based observations that support an additional post-translational regulatory mechanism (oxidation) for Rac1.

2. Rac1 physiology

As a small GTPase, the general function of Rac1 is as a signaling molecule; it responds to various signals through GEF activation, and in its active state, it binds effectors, which ultimately leads to changes in cell motility/morphology, gene expression, cell cycle/survival control, and cell redox status, among other changes (*2*). It is unsurprising that Rac1 deletion is embryonic lethal in mice, as it plays a critical role in a number of biological processes that are fundamental to cell function (Figure 2) (*39*). Research into the physiological function of Rac1 has largely been conducted using Rac1 variants (e.g., constitutively active or inactive) or cell type-specific Rac1 deletion. Recently, a Rac1-specific inhibitor (NSC23766) was discovered, which binds to the cleft between switch I and II, thus inhibiting GEF binding and nucleotide exchange (*40*). This molecule has facilitated pathway-specific research on Rac1 physiology, and it inhibits lamellipodia formation, Nox activation, cell adhesion, and migration (in certain cell types). In this section, I will discuss how Rac1 regulation of cell morphology and gene expression control the critical cell functions cell motility as well as cell cycle and proliferation (*8, 25, 41-45*). The involvement of Rac1 in regulating cell redox status is detailed in section (I)(B), below.

During the cell motility process, Rac1 is found at the leading edge of migrating cells and induces a membrane ruffling phenotype, actin-rich lamellipodia, which form the "motor" that pulls migrating cells forward and are important for haptotaxis and development of cell-cell contacts (*2, 25, 26, 46-54*). Further, cell motility is fundamental

to embryogenesis, epithelial cell renewal, and tissue repair (*53*); for example, Rac1 deletion is embryonic lethal, Rac1-activating glucocorticoids are used to treat inflammatory diseases through enhancing efferocytosis, and Rac1 deletion in vitro and in mice significantly disrupts wound healing in a ROS-dependent manner (*55, 56*). Moreover, certain forms of cell motility require secretion of metalloproteases mediated by Rac1 upregulation of gene transcription (e.g., of MMP1 and MMP2)(*8, 57-65*). Rac1-mediated actin reorganization (most notably through the WAVE and PAK effectors) is a fundamental step in generating intercellular contacts, such as adherens junctions and tight junctions, which are necessary to establishing and maintaining epithelial and endothelial barriers (e.g., the vascular system) (*8, 25, 26, 48, 66-82*). Additionally, cell motility and morphology are essential to immune functions, such as wound healing, pinocytosis, and phagocytosis, all of which are mediated through Rac1 (*26, 83-90*). In addition to the observations that Rac1 is critical to wound healing (above), expression of Rac1 stimulated pinocytosis in fibroblasts; it is required for phagocytosis in leukocytes; FRET-based imaging experiments in macrophages have shown that Rac1 activation and localization are important for phagocytosis, particularly during closure; and Rac1 localization at the membrane in RBL-2H3 cells triggered a phagocytosis system (*50, 91-93*).

It has also been shown that Rac1 regulates the cell cycle and proliferation through cell morphology, ROS generation, and gene transcription (*2, 44, 94-103*). Rac1 binds IQGAP, which that aids in regulating the microtubule dynamics that are responsible for maintaining intracellular organization and mediating cell cycle events (*2, 104-108*). Rac1-mediated NOX activation stimulates cell proliferation in endothelial cells and likely

in ras-transformed fibroblasts (*109-111*). Further, Rac1 activation enhances the production of Cyclin D1, which is necessary for the G1/S cell cycle transition (*112, 113*). As Rac1 is involved in multiple critical cell processes through a variety of mechanisms, it is unsurprising that such processes are often correlated.

3. Rac1 pathophysiology

Because Rac1 is critical to a multitude of essential cell processes, its mutation or dysregulation can produce pathophysiological consequences, including cancer, cardiovascular disease, neurological disorders, and viral infection. The Rac1 effector, PAK1, was shown to be overexpressed or hyperactive in 50% of all breast cancers; the Rac1 splice variant Rac1b has been shown to promote tumorigenesis; and overexpression of Rac1 GEFs (e.g., Trio and P-Rex1 in breast cancer cells) correlates with a poor prognoses in certain cancers (*8, 114-120*). In addition to breast cancer, Rac proteins (Rac1, Rac2, Rac3, and the splice variant Rac1b) have been implicated in leukemia and melanoma as well as testicular, gastric, pancreatic, squamous cell, head, neck, and colon cancers (*8, 116, 121-128*). Typically, Rac1 contributes to pathological conditions when it is dysregulated either through overexpression or enhanced GEF stimulation (*8, 116, 121-128*). However, recently a Rac1 variant (P29S) was identified in almost 10% of the sun-exposed melanomas assayed (20 out of approximately 200), which was corroborated by an independent, smaller-scale study (*129, 130*). Rac1 P29S is self-activating because it maintains a significantly enhanced nucleotide dissociation rate, which in cells produces more activated Rac1 compared with wtRac1 (*131*).

In most cancer-based Rac1 studies, Rac1 is dysregulated in some manner, which is often influenced by the atypical characteristics of tumor microenvironments (e.g.,

ROS generation, hypoxia, enhanced growth factor/cytokine exposure, and inflammation) (*7, 65, 132-143*). Rac1 is a key mediator in cell motility and morphology; thus, it can contribute to metastasis when it becomes dysregulated (*2, 8, 25, 26, 46-52, 57-65*). Rac1 facilitates actin reorganization and upregulated expression/secretion of metalloproteases, both of which are involved in the type of cell motility necessary for tumor metastasis (e.g., epithelial-mesencymal transition) and certain angiogenesis events that can accompany cancer growth (*2, 8, 25, 26, 46-49, 51, 52, 144, 145*). Moreover, Rac1 is critical to multiple redox regulation events through its modulation of Nox (activation) and NOS (expression and activation) as well as its interaction with SOD (*4-6*). As most tumor cells are highly oxidizing environments and redox-active molecules likely contribute to the aberrant signaling events therein, it is unsurprising that such cells support a key role for Rac1 in maintaining tumorigenicity (*146-154*). The microenvironment of the cell (including redox status) can have a huge impact on its proclivity toward tumor transformation, progression, and metastasis (*65, 155-160*). Tumor cells have been shown to produce higher levels of H2O2 and 8-Hydroxy-2'-deoxyguanosine (8-OH-dG), which is an oxidative stress marker (*161, 162*). As discussed above, ROS regulate gene expression as well as cell motility and morphology; thus, it is unsurprising that enhanced ROS generation correlates with the aberrant activity of cancer cells.

In addition to its well-documented role in cancer, Rac1 dysregulation is further involved in cardiovascular diseases, such as atherosclerosis, hypertrophy, atrial fibrillation, and ischemic stroke (*66, 163-169*). Dysregulation of Rac1 in cell motility, morphology, redox regulation, and gene transcription can contribute to such diseases (*66,*

163-169). Rac-mediated signaling contributes to atherosclerosis by mediating cell spreading and contributing to platelet aggregation as well as through excessive ROS generation, which not only maintains the contributing upregulated inflammation signaling pathways but also produces the oxidized lipids that comprise atherosclerotic plaques (*66, 163-165*). The link between Rac1 and cardiovascular hypertrophy is well-established (*66, 166*). Cardiovascular hypertrophy is characterized by increased heart muscle mass, which is facilitated by Rac1 through inflammation-based events, such as through Nox activation, NF-κB-mediated gene transcription, JNK and p38 pathway stimulation, as well as angiotensin II-stimulated Akt signaling (*66, 166-170*). Rac1 activity and ROS are both enhanced in atrial fibrillation models (*66, 171*). Further, a haploinsufficient Rac1 model demonstrated enhanced endothelial barrier maintenance and significantly decreased neuronal death in an ischemia model (*66, 172*).

Rac1 has also been implicated in multiple neurological disorders, including ALS, Alzheimer's disease, and mental retardation, through its role in cell morphology as well as ROS generation (*6, 9, 173-182*). Further, studies have begun to link Rac1 and its role in endocytosis (e.g., phagocytosis and pinocytosis) to viral entry and proliferation sensitivity (*183-186*). Finally, while it is clear that Rac1 is in fact a "Rac of All Trades" with multiple roles in critical cell processes and pathophysiological mechanisms, certain therapeutic strategies have been devised and proposed to mitigate its proclivity toward pathology formation (*2*). For example, Rac proteins require post-translational lipid modification for activity; statins, which inhibit isoprenyl transferases, have been used to inhibit Rac1 activity in heart disease and cancer models (*164, 187, 188*). Interestingly, a recent report proposed a small molecule inhibitor specific for the Rac1/Nox interaction

(*189*). Such specificity in small molecules that target Rac1 interactions in the cell may provide promising new therapies for the plethora of Rac1-mediated pathologies. Clarifying the interactions and post-translational modifications that Rac1 may incur (e.g., oxidation) in its various cell functions may aid in discerning the most specific strategies for targeting diseases. Moreover, understanding redox regulation of Rac1 will inform redox biology, which is increasingly recognized as fundamental but less characterized aspect of cell biology.

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B. Redox regulation of Rac proteins and small GTPases

Oxidation and reduction reactions are important in the numerous signaling pathways that regulate critical physiological processes, and the balance between oxidation and reduction reactions (redox state) is a critical contributor to many diseases. The reactive intermediates (RIs) involved in these processes include ROS, RNS, and reactive thiols. Moreover, protein/RI reactions are diverse and can generate many products. This section will focus on regulation of Rho proteins by reversible thiol oxidation. In this section, I will describe the redox mechanisms involved in regulating Rho GTPases. I will discuss how RIs alter the biochemical properties of these GTPases and the consequences on cell function. I will also discuss evidence for indirect and direct RI regulation of Rho GTPases, highlight cell-based studies that show a strong role for

Rho GTPases in regulating key redox-modulating enzymes, and propose a feedback mechanism for redox status in the cell.

4. RI function, regulation, and production

RIs can be beneficial and harmful in biology. They comprise a number of reactive molecules that can interconvert into other RIs and control physiological processes by interacting with cellular components, including lipids, organelles, proteins, and nucleic acids (*190, 191*). Further, to maintain redox homeostasis in the cell and counterbalance the deleterious effects from RIs, cells have evolved a system of antioxidant molecules and enzymes. Excess RIs can cause changes in the cell redox potential, producing various disease states, such as atherosclerosis, cancer, diabetes, and neurodegenerative disorders. Previous reviews have detailed the chemistry underlying these reactions; I will focus on RI-mediated cysteine oxidation and the effects on Rho GTPases (*10, 192, 193*).

ROS are produced *in vivo* by many enzymes and processes; examples include H_2O_2, $O_2^{\bullet-}$, and hydroxyl radical ($^{\bullet}OH$). ROS can convert into other ROS forms through enzymes, metal-catalysis, and free radical-mediated reactions; for example, in the presence of transition metals, such as Cu^{2+} and Fe^{3+}, the Fenton reaction converts H_2O_2 into $^{\bullet}OH$, a short-lived and highly reactive free radical (*194*). NADPH oxidase (Nox) is a major source of $O_2^{\bullet-}$ in cells, and $O_2^{\bullet-}$ is converted into H_2O_2 by superoxide dismutase (SOD) (*195, 196*). $NO^{\bullet}$ is an important RNS in the cell and is produced by inorganic nitrate reduction and nitric oxide synthase (NOS) (*197*). Nitric oxide modulates multiple cell processes, such as blood pressure regulation, platelet aggregation, and smooth muscle relaxation (*198*). The effects of $NO^{\bullet}$ in vascular disease and cancer biology have been previously reviewed (*199, 200*). While the redox potential of nitric oxide is considered

too low to directly generate thiyl-radical intermediates from protein thiols, its autooxidation product $^{\bullet}NO_2$ can (*192*). Peroxynitrite ($ONOO^-$) is another RNS and can be produced from the direct combination of $NO^{\bullet}$ with $O_2^{\bullet-}$ (*201*). Herein, I discuss mechanisms through which cell-generated NO leads to Rho GTPase activation.

5. RIs regulate Rho GTPases through a reactive Cys in the p-loop GXXXXGK[S/T]C motif

It has been shown that certain Rho GTPases are redox sensitive, and over 50% of Rho GTPases contain a conserved Cys in the p-loop GXXXXGK[S/T]C motif. Based on the Rac1, Cdc42, and RhoA crystal structures, this Cys likely has an altered pKa and is accessible to RIs. Our lab has demonstrated that the p-loop Cys in the Rho GTPases is sensitive to oxidation, which yields an altered activation state (*13*). We have used the radical $^{\cdot}NO_2$ in the presence of reducing agents to generate 500-fold enhanced exchange in Rac1, Cdc42, and RhoA likely through a $1e^-$ (radical-mediated) mechanism. Using redox inactive variants (C18S, Rac1 numbering), our studies have further demonstrated that this radical-mediated nucleotide exchange mechanism operates through redox-active Cys. In a $1e^-$-based mechanism, radicals can transfer their radical to Cys residues. Radicals generally have incredibly short half-lives, and this thiyl radical is typically transient and is transferred to the proximal nucleotide.

Within the Rho GTPase subclass, Rac proteins are also unique in their proximity to and involvement with cell redox-regulating enzymes, such as NOS and NOX (discussed in greater detail below). The addition of H_2O_2 to fibroblasts has been shown to enhance activated Rac1 (*202*). Thus, in Rho GTPases, non-radical oxidants can likely also oxidatively modify the redox-active p-loop Cys, which may promote the activated

form in cells. In contrast with radical-mediated oxidation, non-radical oxidants typically covalently modify Cys thiols and require the thiolate form of the Cys side chain to react, which necessitates an atypically low thiol pKa under physiological conditions.

As mentioned above, the crystal structure for Rac1, Cdc42, and RhoA indicate that this redox-active Cys is not only solvent accessible but also has a lowered pKa. Lowered thiol pKas for Cys residues have been associated with hydrogen bonds, proximity to charged groups, N-terminal residues in an α-helix ("helix-dipole effect"), and solvation effects (*203*). The Rho GTPase redox-active Cys is proximal to multiple charged residues and a divalent metal (Mg^{2+}), located at the N-terminus of an α-helix, as well as in a polar environment. However, evidence suggests that hydrogen bonds are the most likely source of lowered thiol pKas. The Rho GTPase redox-active Cys forms two hydrogen bonds with the bound nucleotide (at the nucleotide α-phosphate and ribose), which supports the hypothesis that this redox-active Cys has an atypically low pKa and is sensitive to 2 e^- (non-radical-mediated oxidative modification) oxidation by non-radical oxidants, such as H_2O_2.

6. In-cell redox regulation of Rho GTPases

Nimnual et al. first described a pathway where ROS production was postulated to alter Rho GTPase activity by modulating Rho regulatory proteins (*204*). Decreased RhoA activity was observed in HeLa cells overexpressing constitutively active Rac1 ($Rac1^{CA}$). This effect was abolished when Rac1 was expressed without its "insert" region. The insert region is likely required for Nox activation and ROS production. $Rac1^{CA}$ depression of RhoA activity was inhibited when cells were treated with the ROS scavenger N-acetylcysteine; conversely, RhoA activity was inhibited when cells were

exogenously treated with 1 mM H_2O_2. Moreover, enhanced p190RhoGAP tyrosine-phosphorylation was observed with downstream Rac1 activation, and tyrosine phosphorylation was abolished in cells pre-treated with an NADPH oxidase inhibitor (diphenylene iodium) as well as cells expressing $Rac1^{CA}$ without the insert region. The authors further showed that activation of Rac1 inactivates a phosphatase (low-molecular-weight protein tyrosine phosphatase, LMW-PTP), which may be upstream of p190RhoGAP. However, interpretation of these results may be complicated by the use of peroxide and N-acetylcysteine at high concentrations, which likely altered the cell redox state, redox targets, and signaling pathways. Further, their hypothesis that Rac1 drives ROS production through Nox to affect RhoA relies on the $Rac1^{CA}$ insert-deletion mutant (*204*). The importance of the insert region in Nox activation has been debated, and at least two reports describe the insert region as dispensable to ROS production (*205, 206*). However, Nox and Rac isoforms are expressed in different cell types and bind with varying efficiencies, which may account for this discrepancy, and downstream effects from $Rac1^{CA}$ may play a role. An alternative approach to insert-deletion mutants is necessary to interpret the mechanism for ROS regulation of RhoA.

The Burridge lab has recently shown that exogenous and endogenous ROS can stimulate Rac1 activity in fibroblasts (*202*). Expanded studies were performed with the related Rho GTPase RhoA and showed that activation and stress fiber formation were reversible by washing out peroxide and abolished upon N-acetylcysteine treatment. Further, ROS activation of RhoA was abolished when two critical cysteines were mutated (RhoA C16A/C20A; in RhoA, the redox active Cys is Cys20, and the analogous Cys in Rac1 is Cys18). Notably, RhoA C16A/C20A responds normally to GEF-mediated

activation and C3 toxin inactivation. These results show that ROS can directly activate RhoA (and likely Rac1) through a mechanism involving the critical cysteine Cys20 (Cys18 in Rac1).

7. Rho GTPases control redox-regulating enzymes

Rho GTPases play a key role in regulating cell redox status; they can modulate cell redox status in response to molecular and mechanical forces as well as an altered redox state. This response is particular to cell type, subcellular location, and initiating event; thus, the downstream effects are diverse and highly regulated. In cell-based and animal experiments, Rac proteins (Rac1 and Rac2 isoforms) and RhoA regulate expression and activity of primary redox-modulating enzymes in the cell, Nox ($O_2^{\bullet-}$ generation), NOS ($NO^{\bullet}$ generation), and SOD ($O_2^{\bullet-}$ dismutation into H_2O_2) (Figure 1.3). As RIs can modulate the activity of redox sensitive Rho GTPases in cell-based studies and facilitate guanine nucleotide dissociation *in vitro*, these GTPases are likely involved in a redox feedback loop.

i. Rac proteins and RhoA regulate NOS expression and activity

The activity and expression of the three NOS isoforms, eNOS, nNOS (neuronal NOS), and iNOS (inducible NOS), are highly regulated (*207*). Whereas Rac proteins have been shown to regulate NOS expression and activity, RhoA regulates NOS expression (Figures 1.3 and 1.4). Further, NOS bioavailability is, in part, attributed to its mRNA stability, which Rho GTPases can either enhance (Rac1) or reduce (RhoA) (*208, 209*). Rac1 promotes eNOS transcription through PAK1 (p21-activated kinase), and RhoA reduces mRNA stability through ROCK (Rho-associated protein kinase) (*210*). Moreover, Rac1 and RhoA work in opposition to regulate NOS activity through the

PI3K/Akt redox-sensitive pathway; RhoA inhibits this pathway, and Rac1 activates it (*210, 211*). Notably, Ras also modulates the PI3K/Akt pathway to regulate eNOS activity (*212*). As further evidence of RhoA/Rac1 crosstalk in NOS activation, Rac1 regulates cGMP-dependent kinase, which inhibits RhoA activation (*210*). The Rac1/PAK1 interaction further enhances eNOS activity by stimulating cell uptake of L-Arginine (*210*). Rac proteins may regulate NOS function in two additional ways, controlling localization and direct protein-protein interaction (*5, 213*). Rac2 and iNOS interact in a direct, GTP-independent manner, and Rac2 overexpression promotes iNOS localization to cytoskeletal complexes (*213*). Rac1 has been shown to directly interact with all of the NOS isoforms, and overexpression of $Rac1^{CA}$ enhances eNOS and nNOS activity, likely through direct interaction (*5, 208, 213*). As NOS isoforms interact with Rac isoforms with varying efficiencies depending upon cell type, stimulating event, and Rac nucleotide status, this interaction may aid in proper compartmentalization for NO-generation.

ii. Rac proteins activate Nox

Rac proteins regulate Nox activity, as they can directly interact with and activate multiple Nox isoforms to produce $O_2^{\bullet-}$, including Nox1, 2, 3, and 4 (*4*). Rac1 is required for Nox1 activity in vascular smooth muscle cells, and Rac2 activates Nox2 in neutrophils (*214, 215*). Interestingly, Rap1A, an NKCD-motif-containing Ras subclass GTPase, has also been implicated in Nox activation (*215*). Nox activity can be stimulated by a multiple factors, including shear stress, angiotensin II, thrombin, insulin, and VEGF (vascular endothelial growth factor) (*196, 216, 217*). Various models have been proposed for Rac activation of Nox, and data support Nox activation by GTP-bound Rac and GDP-bound Rac complexed to GDI (GDP dissociation inhibitor); the authors suggest that GDI

association maintains the active Rac conformation even when GDP-bound, as Rac likely binds Nox in this conformation (*214, 218*). The common steps in the models for Rac1 activation of Nox are as follows: first, upon stimulation, Rac and the cytosolic Nox components translocate simultaneously, but independently, to the cell membrane (*214, 215*); next, likely through its switch I and insert motifs, Rac directly binds p67 to activate Nox (*214*). Controversial in these models is whether Rac1 also directly interacts with flavocytochrome b558, whether it activates Nox either through an adaptor function or mediates electron transfer during $O_2^{\bullet-}$ production, and whether the Rac insert region is required for Nox activation (*214, 215*). Depending on cell type, localization, and stimulating event, Rac-dependent Nox activation can promote various signaling pathways with physiological and pathological consequences, such as transcription activation, inflammatory responses as well as cardiovascular and neurological diseases (*209*).

iii. Rac1 directly interacts with SOD in a nucleotide- and redox-dependent manner

Recent evidence supports a role for Rac1 in regulating SOD1 (Figure 1.3), a key antioxidant enzyme in the cell. In a detailed study, Harraz et al. showed that Rac1 interacts with SOD1 in a nucleotide-dependent manner, indicative of GTPase/effector interactions (Figure 1.4) (*219*). As this study involved cell-based assays, our lab is using *in vitro* methods to quantitate this interaction and have validated its nucleotide dependence (unpublished observations). Harraz et al. showed that cell redox conditions also affect the Rac1/SOD1 interaction and suggested a feedback mechanism. However, the question remains whether the Rac1/SOD1 interaction enhances SOD1 activity, as in Nox and NOS. Interestingly, these experiments were performed in an Amyotrophic

lateral sclerosis (ALS) model, and SOD mutations that are common in ALS patients were proposed to uncouple the Rac1/SOD feedback mechanism and enhance cell toxicity. A redox regulatory role for the Rac1/SOD interaction in neural physiology and pathology is consistent with Rac regulation of NOS and Nox in key physiological and pathological pathways.

I have reviewed the in vitro and cell-based literature demonstrating that RIs regulate Rho GTPase activity and certain Rho GTPases, such as the Rac proteins, control the activities of the predominant redox-modulating enzymes in cells. I speculate that Rac is a common control element for regulating the cell redox state. Moreover, RhoA has been shown to play a key role in regulating NOS. I postulate that cell redox conditions (oxidative/reducing) regulate the activity of these GTPases; however, the role of these GTPases in controlling RI-regulating enzymes suggests a potential feedback mechanism.

8. Conclusions and future directions

I have discussed the importance of RIs in modulating the activity of Rho GTPases and the role of these GTPases in controlling the expression and activity of RI-regulating enzymes. Our data and a growing body of literature suggest that Rho GTPases are particularly susceptible to redox control given their unique redox-sensitive cysteine-containing p-loop motif. This redox-sensitive cysteine interacts with the bound nucleotide, and $1e^-$ and $2e^-$ redox modification at this cysteine enhances nucleotide exchange, likely by disrupting these interactions. Under physiological conditions, RIs likely activate Rho GTPases, whereas oxidative stress may promote their inactivation. To aid in discerning whether RI regulation of Rho GTPases is direct or indirect, I have identified a redox insensitive variant that mimics wtRac1. Moreover, cell-based studies

should be performed with endogenous RI sources, redox state characterization, and absent GTPase overexpression. Further, the Rho subclass also regulates expression and activity of key redox regulating enzymes. RhoA regulates NOS expression and activity. Rac controls NOS and Nox, and Rac1 has recently been shown to directly interact with SOD1. Given that Rac1 and SOD1 are ubiquitously expressed proteins involved in numerous cell functions, future experiments should be directed to discerning the mechanism of and effects from this interaction. Further, as the literature is increasingly dedicated to redox-based signaling, perhaps future studies will better define how RIs regulate Rho GTPase activity and whether these GTPases are regulated by or control additional redox-modulating enzymes. Herein, I demonstrate that Rac1 Cys18 has an altered pKa that mediates redox-modification at this site, which sufficiently enhances intrinsic dissociation. Moreover, I can show that Rac1 and SOD interact in vitro, which further supports and expands the role of Rac1 in regulating the cell redox status.

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Figure 1.1.* GTPase nucleotide cycle. GTPases are active in the GTP-bound form. GAPs (GTPase activating proteins) promote GTP hydrolysis, which results in the inactive, GDP-bound form. GEFs (guanine nucleotide exchange factors) facilitate nucleotide dissociation, and, as the in-cell GTP:GDP ratio is typically high (greater than or equal to 10:1), GTPases bind GTP by mass action and become activated. GDIs can also regulate Rho GTPases by sequestering them, which prevents nucleotide exchange and membrane insertion. We hypothesize that physiological levels of RIs facilitate exchange, which typically populate GTPases in the active conformation, whereas oxidative stress conditions inactivate the GTPases. RhoA is shown in green. The regions that undergo nucleotide-dependent changes in conformation are highlighted in red (switch I) and magenta (switch II). GEF is indicated in yellow, GAP is colored orange, and GDI is in teal. The following structures (PDB ID) were used to generate this model: RhoA-GTP (1A2B), RhoA-GTP with a GAP (1OW3), RhoA-GDP (1FTN), RhoA-GDP complexed with GDI (1CC0), and RhoA-GDP complexed with a GEF (1LB1).

Figure 1.1

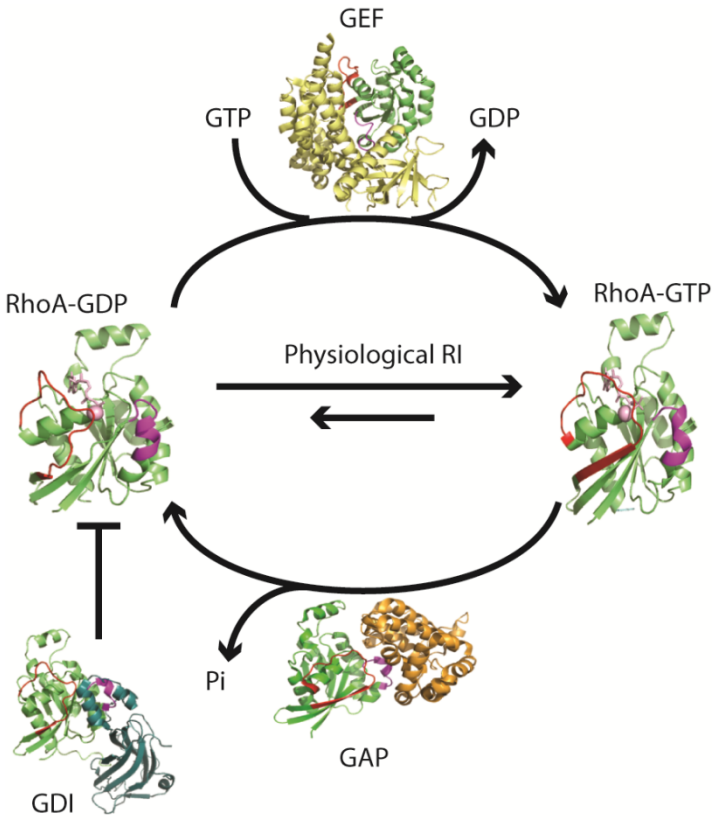


Figure 1.2: Most commonly studied Rac1 effectors and downstream effects of Rac1/effector interaction. The Rac1 nucleotide switch mechanism is indicated. When Rac1 is GTP-bound, the top set of arrows indicated effector interactions that can result (*2*). The bottom set of arrows indicate the specific outcome from the indicated Rac1/effector interaction.

Figure 1.2

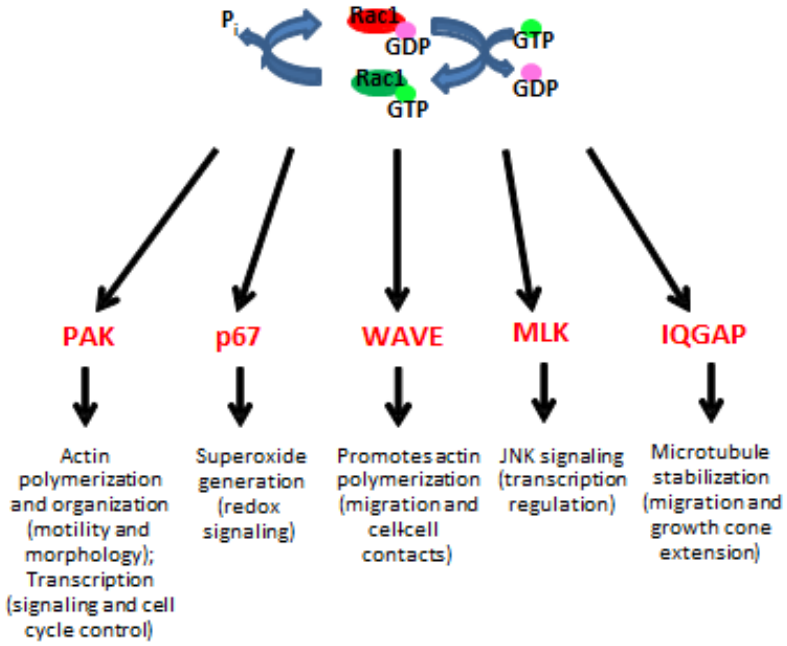

Pi
Rac1
GDP
Rac1
GTP
GTP
GDP
PAK
p67
WAVE
MLK
IQGAP
Actin polymerization and organization (motility and morphology); Transcription (signaling and cell cycle control)
Superoxide generation (redox signaling)
Promotes actin polymerization (migration and cell-cell contacts)
JNK signaling (transcription regulation)
Microtubule stabilization (migration and growth cone extension)

Figure 1.3: * Rac directly interacts with Nox, NOS, and SOD. Rac isoforms have been shown to directly enhance the activities of NOS and Nox isoforms (*4, 5, 208*). In several Nox isoforms, Rac must interact with the p67 subunit (and possibly flavocytochrome b558) for Nox to convert O_2 to $O_2^{\bullet-}$ (*4, 214*). In addition to evidence that Rac indirectly stimulates NOS activity, Rac1 has been shown to increase eNOS and nNOS activity through direct interaction, aiding in the conversion of O_2 to NO (*5, 208*). Finally, recent data shows that Rac1 directly interacts with SOD1 in a nucleotide- and redox-dependent manner (*219*).

Figure 1.3

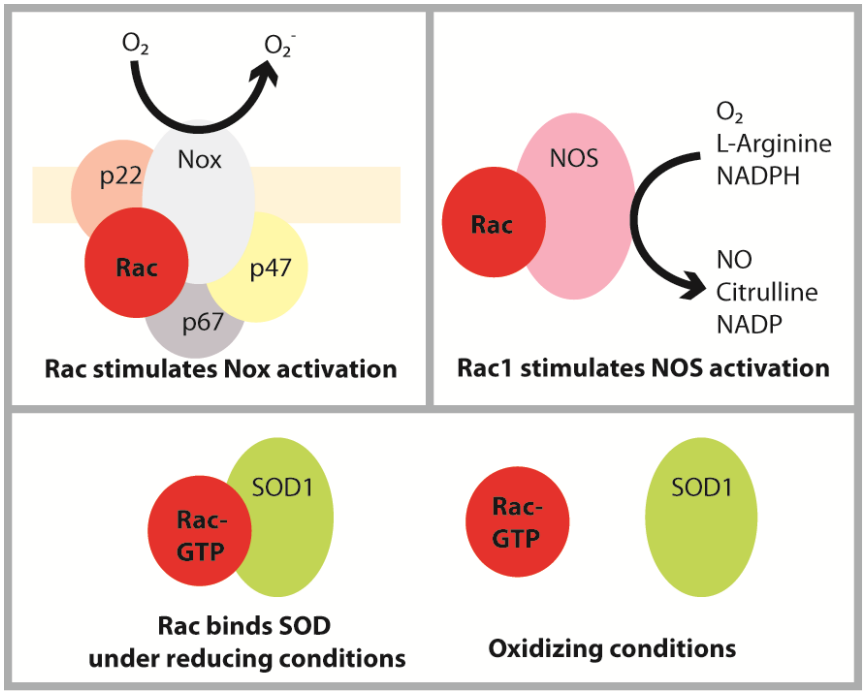
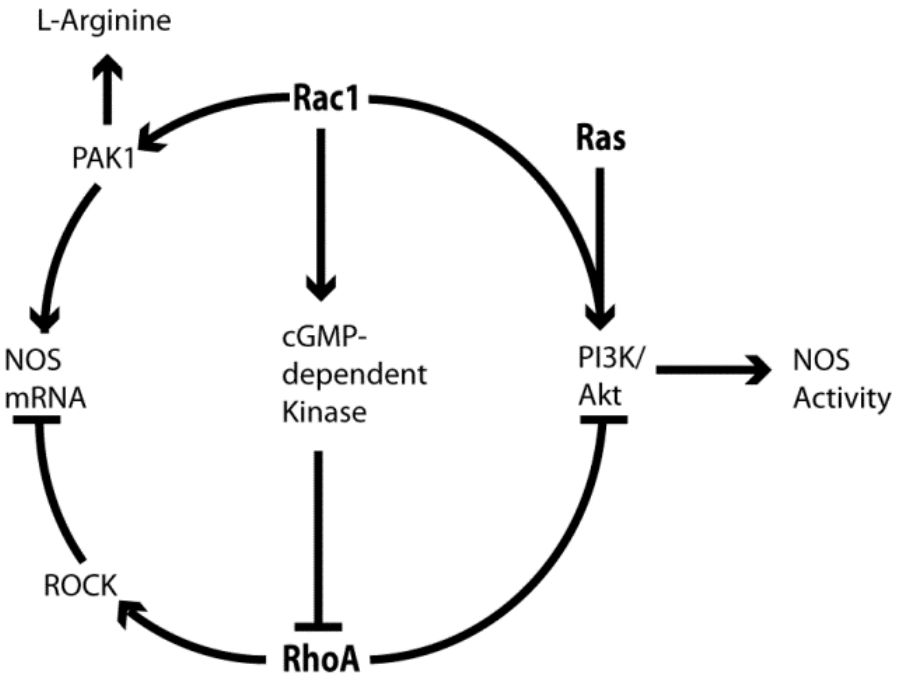

O_2
O_2^-
Nox
p22
Rac
p47
p67
Rac stimulates Nox activation
NOS
Rac
O_2
L-Arginine
NADPH
NO
Citrulline
NADP
Rac1 stimulates NOS activation
SOD1
Rac-
GTP
Rac binds SOD
under reducing conditions
Rac-
GTP
SOD1
Oxidizing conditions

Figure 1.4: * GTPase crosstalk in NOS regulation. Shown below, RhoA regulates NOS expression by decreasing mRNA stability through ROCK, and it decreases NOS activity through inactivation of the PI3K/Akt pathway (*208-210*). Rac1 enhances NOS mRNA expression through PAK1, and it increases NOS activity by direct interaction (*5, 208, 220*), activating the PI3K/Akt pathway (*211*), increasing cell uptake of L-Arginine, and downregulating RhoA activity through cGMP-dependent Kinase (*210*). Ras also plays a role in NOS stimulation through the PI3K/Akt pathway (*212*).

Figure 1.4


L-Arginine
Rac1
PAK1
Ras
NOS
mRNA
cGMP-
dependent
Kinase
PI3K/
Akt
NOS
Activity
ROCK
RhoA

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I. Redox regulation of Rac1

A. Overview

The Rac1 GTPase is a guanine nucleotide binding protein that regulates multiple cell processes including cell cycle, gene expression, cell motility and morphology, as well as redox homeostasis and signaling. Rac1 deletion is embryonic lethal, and its dysregulation or mutation can promote cancer, cardiovascular disease, and neurological disorders. The Rac1 activation state is nucleotide dependent; GTP-bound Rac1 produces the activated conformation that facilitates effector binding, whereas GDP-bound Rac1 is believed inactive. Rac1 activity is highly regulated by modulatory proteins and post-translational modifications. Rac1 interacts with several effectors and propagates downstream signaling through numerous pathways. While much attention has been devoted to guanine nucleotide exchange factors that act on Rac1 to promote GTP loading and Rac1 activation, cellular oxidants can also regulate Rac1 guanine nucleotide exchange. Herein, we show Rac1 contains a redox active cysteine (Cys18) that can be selectively oxidized at a physiological pH because it has a lowered pKa. Oxidation of this cysteine perturbs guanine nucleotide binding and promotes guanine nucleotide dissociation. In cells, a sulfonic acid oxidation variant at this site, Rac1 C18D, is significantly more activated compared with wtRac1 and a redox insensitive variant, Rac1 C18S. Our results suggest that Rac1 cysteine oxidation is a novel post-translational modification that regulates Rac1 activation and signaling.

B. Introduction

Rac1 is a ubiquitously expressed guanine nucleotide binding protein in the Rho subclass of the Ras superfamily of GTPases. It is involved in critical cell functions and processes, such as cell motility and morphology, gene expression, cell cycle control, as well as redox regulation signaling and homeostasis (*2*). Rac1 is essential because its deletion causes embryonic lethality (*39*). Moreover, dysregulation or mutation of Rac1 can cause cancer, cardiovascular disease, and neurological disorders (*6-9, 39, 66, 114*). Given its critical role in cell physiology and human disease, it is not surprising that Rac1 activity is tightly regulated. Rac1 populates an activated conformation when it is bound to GTP and shifts to an inactive conformation when GTP is hydrolyzed, and it is bound to GDP (*1, 20*). GDP must be released in cells for Rac1 to again bind GTP and become activated; Rac1 binds GTP and GDP with similar affinity, but the ratio of GTP:GDP in cells is typically at least 10:1 (*19, 20, 27*). While intrinsic rates of GDP release and GTP hydrolysis are too slow for temporal regulation of Rac1, Rac1 activity can be up-regulated by modulatory proteins GEFs (guanine nucleotide exchange factors) that facilitate GDP release and exchange of GDP for GTP, and Rac1 is down-regulated by GAPs (GTPase activating proteins) that catalyze GTP hydrolysis (*20, 27*). Further, as a Rho GTPase, Rac1 is also regulated by GDIs (guanine nucleotide dissociation inhibitors), which prevent Rac1 association with the membrane (*29, 30*). Additionally, Rac1 can undergo various post-translational modifications, including C-terminal lipid modification, phosphorylation, ubiquitination, and SUMO-lation (*32, 33, 36, 38*) to regulate its spatial and temporal activity. We demonstrate herein that oxidative modification is likely an additional mechanism for regulating Rac1 activation.

Rac1 plays an important role in regulating redox signaling and homoeostasis. It binds to and directly activates multiple isoforms of Nox (NADPH oxidase) enzymes, which produce superoxide in many cell types and tissues (*4*). Typically, superoxide has an extremely short half-life and can be converted to the less reactive signaling molecule hydrogen peroxide by superoxide dismutase (SOD) (*195, 196*). Rac1 further binds to and regulates the activity of one of the predominant reactive nitrogen species (RNS) producers in the cell, NOS (nitric oxide synthase) (*5, 208-213*). NOS generates nitric oxide, which involved in multiple fundamental physiological processes (*197*). While NO is best known as a vascular relaxation factor, it can function as a neurotransmitter and is used in pathogen defense (*221-223*). Recently, it was reported that Rac1 interacts with SOD1 (superoxide dismutase) in a redox- and nucleotide-dependent manner; SOD1 a major antioxidant enzyme, which converts the highly reactive superoxide to the less reactive hydrogen peroxide (*6*). Hydrogen peroxide can react with biomolecules in the cell to alter signaling networks; however, it can also be destructive at oxidative stress concentrations (*224*).

Given that Rac1 colocalizes with and modulates the activities of central redox enzymes, we investigated the effect of oxidative modification on Rac activity *in vitro* and in cells. We previously showed that NO_2 and H_2O_2 enhance GDP dissociation in Rac1, which likely promotes the activated form of the protein in vivo (*13, 225*). Further, we have shown that treatment of HeLa cells with H_2O_2 causes Rac1 activation (*202*). Thus, we wanted to further investigate redox-dependent modulation of Rac1 activity. Herein, we demonstrate *in vitro* and in cells that oxidative modification of Rac1 promotes its activation. We find that Rac1 contains a redox-sensitive cysteine in a conserved

phosphoryl binding motif that interacts with the bound nucleotide, which possesses a lowered pK_a. This cysteine can be site-selectively oxidized with glutathione, which impairs interaction with the bound nucleotide and enhances GDP dissociation. Moreover, we generated and characterized a redox-insensitive Rac1 variant (Rac1 C18S) and an oxidation (sulfonic) mimetic (Rac1 C18D). Rac1 C18S could not be oxidized, and its biochemical characteristics were similar to wtRac1. However, similar to glutathiolated Rac1, Rac1 C18D had a significantly enhanced GDP dissociation rate (greater than 200-fold). Further consistent with our in vitro findings, in HEK-293T cells, expression of a sulfonic acid oxidation variant at this site, Rac1 C18D, is significantly more activated compared with wtRac1 and the redox-insensitive variant Rac1 C18S.

C. Methods and Materials

1. Expression and purification of recombinant proteins

Human wtRac1 (1-188, C178S) and Rac1 Cys18 variants were expressed in the pET 15b vector system (EMD Millipore) or the pQlinkHG vector system (Addgene). Escherichia coli BL21 (DE3) RIPL cells (Strategene) were transformed with the Rac1 expression vector, and grown at 37°C to 0.6 $O.D._{600}$, and Rac1 expression was induced upon addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 4 more hours at 37°C, the cells were centrifuged, and the cell pellet was lysed through sonication in 50 mM KH_2PO_4 pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 10 μM GDP, and 5 mM β-mercaptoethanol (BME). Rac1 proteins were purified using a Ni-NTA column (Qiagen) to >95% purity by SDS-PAGE. Rac1 was stored in 50% glycerol at -20°C. Human p50RhoGAP was expressed in the pQlinkH vector system (Addgene), and human Tiam1 DH/PH was expressed in the pET28s vector system; E. coli BL21 (DE3) RIPL cells were

transformed with either protein and grown at 37°C to 0.6 $O.D._{600}$. Tiam1 and p50RhoGAP were lysed in 20 mM Na_2HPO_4 pH 7.4, 150 mM NaCl, 20 mM Immidazole, and 5 mM BME; purified using a Ni-NTA column (Qiagen); and stored in 50% glycerol.

2. Glutathiolation of Rac1

Oxidized glutathione was added to Rac1 protein at 1000-fold excess for 15 min at 37°C in 50 mM KH_2PO_4 pH 6, 150 mM NaCl, 5 mM $MgCl_2$, 50 μM GDP, and 0.1 μM DTPA (glutathiolation buffer). The sample was then dialyzed against pre-chilled glutathiolation buffer for 20 hours. This procedure was repeated 3 times. The relative levels of glutathiolated and non-glutathiolated Rac1 were confirmed by gel-shift using non-reducing SDS-PAGE, which correlated with relative signal intensities obtained from electrospray chromatography (ESI-MS) analyses. For MS, Rac1 was desalted using C18 reversed-phase chromatography and directly injected into an LTQ-Orbitap XL (Thermo Scientific).

3. Cys18 Reactivity with 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-f)

wtRac1 and Rac1 C18S were reduced in 50 mM KH_2PO_4 pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 10 μM GDP, and 1 mM DTT. They were then buffer exchanged through dilution and concentration using a centrifugal filter device (10 kDa molecular weight cut-off, Millipore) to 100 μM in 50 mM KH_2PO_4 pH 6 and 150 mM NaCl. The protein was then diluted to 10 μM in 50 mM KH_2PO_4 and 150 mM NaCl at a range of pH values, and 7.5 μM ABD-f (Anaspec) was added to initiate the reaction. ABD-f fluorescence is significantly enhanced upon reaction with Rac1 (excitation 389 nm, emission 513 nm); the reaction rate was monitored using a Spectromax M5 spectrometer (Molecular

Devices). The reaction rates were quantified using a single exponential (Prism 3.03, Graphpad), and the Rac1 C18S reaction rates were subtracted from the wtRac1 reaction rates to generate the Cys18 reactivity curve.

4. GDP Dissociation Assay

Rac1 was preloaded with 2'-/3'- O- (N'- Methylanthraniloyl)guanosine- 5'- O- diphosphate (mant-GDP; Biolog) by incubating 10-fold excess mant-GDP with Rac1 in 20 mM Tris pH 7.5, 50 mM NaCl, 200 mM $(NH_4)_2SO_4$, and 0.1 mM EDTA for 1 hr at 37°C. The reaction was then incubated on ice upon addition of 20 mM $MgCl_2$ for at least 1 hr. Excess nucleotide was removed by buffer exchange (20 mM Tris pH 7.5, 150 mM NaCl, and 5 mM $MgCl_2$). GDP was then added at1000-fold excess, and the rate of GDP dissociation was determined by exciting at 365 nm and monitoring the fluorescence emission at 435 nm using an LS 50B fluorescence spectrometer (Perkin Elmer). The data were normalized and fit to a single exponential using Prism 3.03 (n=3).

5. Rac1 Thermal Stability

Rac1 thermal stability was measured using fast Quantitative Cysteine Reactivity (fQCR). Rac1 was diluted to 2 μM in 100 mM K_2HPO_4 pH 7 and incubated with 1 mM ABD-f for 3 min over a range of temperatures. The reaction was quenched on ice with 0.1 N HCl, and the fluorescence was measured using a PHERASTAR spectrometer (excitation 389 nm and emission 513 nm; BMG Labtech). The data were normalized and fit to a Boltzmann sigmoidal function to determine T_m (the temperature at which half the protein population is unfolded; Prism 3.03; n=3).

6. GTP Hydrolysis

Rac1 single turnover GTP hydrolysis rates were determined in the absence and presence of p50 RhoGAP (1:1000 GAP:Rac1)) as previously described, with the following modifications (*226*). Inorganic phosphate was removed from all buffers by adding 1 mM inosine to each buffer and dialyzing 1 unit nucleoside phosphorylase in the buffers. Rac1 was then loaded with GTP by incubating with 10-fold excess GTP at 37°C for 1 min in 20 mM HEPES pH 8, 20 mM $(NH_4)_2SO_4$, 1 mM EDTA, 0.1 mM DTPA, and 1 mM inosine; the excess GTP was removed using a PD-10 column (GE Healthcare). The reaction was performed in 20 mM Tris pH 7.4, 50 mM NaCl, 0.1 mM EDTA, and 0.1 mM inosine. GTP hydrolysis was initiated by adding 100 μM $MgCl_2$ to a sample containing 50 μM Rac1 and Flippi 5U (Addgene). Flippi undergoes a conformational change upon binding inorganic phosphate, which also alters the fluorescence resonance energy transfer (FRET) signal that Flippi produces. Therefore, Rac1 GTP hydrolysis rates were determined by monitoring the change in FRET signal using a Spectramax M5 spectrometer (excitation 415 nm and the ratio for emission at 475 and 515 nm). The data were normalized and fit to a single exponential (Prism 3.03; n=3).

7. NMR experiments

Rac1 was expressed and purified as described above except that the medium supplied to the E. coli cells during expression was ^{15}N-enriched M-9 minimal media. Two dimensional ^{1}H-^{15}N HSQC NMR experiments were performed using a Varian Inova 700 MHz spectrometer with a cryoprobe at 25°C in 50 mM Tris Malate pH 6.8, 50 mM NaCl, 5 mM $MgCl_2$, 50 μM GDP, 0.1 mM DTPA, and 1 mM DTT (DTT was not added to the glutathiolated sample). The Rac1 concentration was 200 μM.

8. Cell lines, plasmids and reagents

HEK-293T cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Sigma) and maintained at 37°C in 5% CO_2. Mammalian expression constructs with full-length human Rac1 and the C18S variant in the pCMVJ3 vector system were gifts from Keith Burridge, UNC; pCMVJ3-Rac1 C18D was generated from wtRac1 by using PCR-based mutagenesis. The tert-butyl H_2O_2 was from Aldrich.

9. PAK pulldowns

Levels of active, GTP-bound Rac1 were assessed by pulldown assays using the p21-binding domain of PAK1 (GST-PAK-PBD, gift of Keith Burridge) as described previously (*227*). Briefly, HEK-293T cells were transiently transfected with Rac1 expression plasmids using the TransIT transfection reagent (Mirus) according to the manufacturer's instructions. The next day or at 80-90% confluency, the cells were starved in serum-free DMEM for 3 h. The cells were then washed twice with ice-cold phosphate-buffered saline (pH 7.4) and lysed in magnesium lysis buffer (MLB) with protease inhibitors. Equal volumes were removed from each lysate for total protein analysis. To each lysate, glutathione-agarose beads containing 20 μg of GST-PAK-PBD were added and incubated at 4°C with rocking for 60 min. Agarose-GST-PAK-PBD and associated Rac1 were pelleted and washed three times with 500 μl wash buffer (25 mM Tris pH 7.5, 40 mM NaCl, and 30 mM $MgCl_2$). The final pellets were resuspended in 1× protein sample buffer and resolved on SDS-PAGE. Rac1 constructs were detected by immunoblotting using anti-HA antibody (Roche). Equal protein loading was confirmed

with anti-tubulin (Sigma-Aldrich), and the Rac1 C18S as well as C18D western blots were normalized to wtRac1.

D. Results

Rac1 interacts with multiple redox enzymes, including NOS, NOX, and SOD1, and it directly activates NOX isoforms 1, 2, and 4 (*4-6*); further, evidence suggests that Rac1 may also directly activate NOS (*5, 213*). We previously showed that Rac1 activity can be directly modulated by RNS and ROS (*13, 225*). Our earlier work focused on radical-mediated regulation of Rac1 activity, wherein we found that redox agents capable of thiyl radical formation at Rac1 Cys18 oxidized the guanine base, resulting in enhanced nucleotide exchange. We sought to expand on these studies by examining whether non-radical mediated thiol oxidation of Rac1 can alter its activity. As Rac1 Cys18 is located in the nucleotide binding pocket and forms multiple hydrogen bonds with the nucleotide (*228*), we postulated that the thiol side chain may have an altered pKa, rendering it sensitive to oxidation. Moreover, oxidation of this thiol could alter guanine nucleotide binding and, consequently, Rac1 activity.

1. Rac1 Cys18 is selectively oxidized at a physiological pH because it has a low pK_a

We first examined cysteine reactivity for Rac1 using a thiol-modifying reagent, ABD-f, that preferentially reacts with the thiolate form of cysteine (*229*). Cysteine oxidation of Rac1 was assessed by measuring the ABD-f fluorescence for wtRac1 and the redox-insensitive variant Rac1 C18S from pH 5.6 to 7.6 (*229*). The wtRac1 fluorescence was subtracted from Rac1 C18S and normalized to produce a pH titration curve for Rac1 Cys18 (Figure 2.1B). The estimated pK_a for the Cys18 thiol is 6.8, which is almost 2 pH

units lower than typical free cysteine pK_a range that varies from 8-9 (*203, 230-232*). The Rac1 construct under investigation (Rac1 1-188, C178S) contains 5 cysteines, some of which are partially accessible. However, our data shows that Cys18 is the only cysteine that appreciably reacts with ABD-f at a physiological pH, as Rac1 C18S shows minimal reactivity with ABD-f over the pH range investigated (5.6-7.6; Figure 2.1A). These results indicate that Rac1 Cys18 has a depressed pK_a, which renders it particularly reactive to oxidation under physiological conditions.

To examine whether Rac1 Cys18 can be selectively oxidized by physiological oxidants, we treated Rac1 with oxidized glutathione and used LC-ESI-MS to determine the site(s) of oxidation in our Rac1 construct. WtRac1 and Rac1 C18S were treated with oxidized glutathione using 1000-fold excess GSSG:Rac1 at 37°C for 15 min at pH 6. The sample was then cooled to 4°C, excess glutathione was removed by dialyzing the reaction sample in pre-chilled buffer overnight, and analyzed via LC-ESI-MS using a C18 reverse phase column. In Figure 2.2C and 2.2D, the MS spectra for unmodified and glutathiolated wtRac1 are shown, and Figures 2.2A and 2.2B display spectra for unmodified and glutathiolate-treated Rac1 C18S, respectively. Only the MS spectrum for glutathiolated wtRac1 (Rac1 S-SG) shows a molecular weight peak shift of 305 Da (the expected mass shift for glutathione addition; Figure 2.2D). To further enrich the Rac1 S-SG sample, we used an iterative approach in which the glutathiolation reaction was successively performed 4 times. Consistent with enrichment of Rac1 S-SG, an enhanced ESI-MS signal at the 305 Da over the Rac1 molecular weight was observed for the enriched Rac1 S-SG sample (Figure 2.2D, inset). These results indicate that Rac1 can be selectively oxidized by GSSG at physiological pH values, consistent with its altered pK_a.

2. Oxidation of Rac1 Cys18 significantly enhances the intrinsic rate of GDP dissociation

We have previously shown that NO-mediated oxidation of Rac1 Cys18 can alter Rac1 activity by promoting guanine nucleotide dissociation (*225*). We also showed that reaction of Rac1 with H_2O_2 can enhance the rate of GDP dissociation 10-fold, suggesting that non-radical oxidation of Rac1 can also alter Rac1 activity (*13*). Given these observations, we explored whether oxidative modification by GSSG could modulate Rac1 guanine nucleotide binding. Typically, Rac1 binds GDP and GTP with high affinity, and, consequently, GDP dissociation is very slow (*13, 233*). Hence, given the similar affinity of GTP and GDP and the GTP:GDP ratio in cells (>10:1); nucleotide release primarily yields the activated GTP-bound form of Rac1 (*20, 27*). For these experiments, we employed the redox-inactive variant Rac1 C18S as a negative control and Rac1 C18D as a sulfonic acid oxidation mimetic. To discern whether mutation or oxidation of Rac1 at position 18 alters guanine nucleotide binding, we measured the rate of GDP dissociation for wtRac, Rac1 C18S, Rac1 C18D, and Rac1 S-SG. Rac1 was pre-loaded with fluorescently labeled GDP (mant-GDP), and Rac1 GDP dissociation was measured through a decrease in mant-GDP fluorescence as a function of time (Figure 2.3) (*234*). In addition, the rate of GDP dissociation was measured in the presence of the Rac1 GEF Tiam1. The data were fit to a single exponential. As shown in Table 1, wtRac1 and the redox-insensitive control Rac1 C18S have GDP dissociation rates of approximately 1 x 10^{-4} s^{-1}, consistent with previous observations (*13*). Moreover, both wtRac1 and C18S were similarly responsive to GEF-mediated GDP dissociation. Thus, mutating Rac1 Cys18 to a Ser residue does not significantly alter nucleotide dissociation kinetics nor does it perturb GEF-mediated guanine nucleotide dissociation. In contrast, oxidative

modification of Rac1 by glutathione at Cys18 produces a dramatic enhancement (200-fold) in the rate of GDP dissociation. Intriguingly, we also observe a similarly enhanced rate of GDP dissociation for the Rac1 C18D variant, suggesting that C18D may mimic the effects of a sulfonic acid or glutathione modification. For Rac1 C18D and Rac1 S-SG, GDP dissociation was approximately 200-fold enhanced. These results indicate that Rac1 Cys18 is sensitive to oxidation, and oxidative modification at this residue greatly enhances the intrinsic rate of guanine nucleotide dissociation.

3. Rac1 Cys18 oxidation does not alter protein stability or the rate of GTP hydrolysis in the presence or absence of GAPs.

Given the significant increase in GDP dissociation observed for Rac1S-SG and Rac1 C18D, we evaluated whether either Rac1 stability was also affected by oxidative modification. fQCR was used to measure thermal stability (*229*), by monitoring ABD-f reactivity as a function of temperature. As the protein unfolds, any buried Cys residues are exposed and ABD-f can react. The wtRac1, Rac1 C18S, and Rac1 C18D melting temperature (T_m) values were compared as shown in Figure 2.4A. Rac1 C18D retained the thermal stability of wtRac1; both Rac1 C18D and wtRac1 had T_m values at 55°C. Interestingly, the redox-insensitive variant Rac1 C18S demonstrated enhanced thermal stability with a T_m value at 60°C. Thus, while Rac1 oxidation may promote guanine nucleotide exchange and Rac1 activation in cells, the sulfonic acid mimetic (C18D) does not destabilize Rac1.

In-cell regulation of Rac1 activity involves both exchange of GDP for GTP to activate Rac1 and GTP hydrolysis, which inactivates the protein. To fully characterize the effect of Rac1 Cys18 oxidation, we performed a GTP hydrolysis assay with and without the Rac1 GAP p50RhoGAP using wtRac1, Rac1 C18S, and Rac1 C18D. We pre-loaded

Rac1 with GTP and measured the rate of phosphate release using a PBP (phosphate binding protein) construct, Flippi 5U (*235*). Flippi 5U undergoes a conformational change upon binding inorganic phosphate; the Flippi 5U construct includes green and yellow fluorescent proteins (GFP and YFP, respectively), which produce a significantly reduced FRET signal upon phosphate binding through a conformational change (*235*). Therefore, the rate of GTP hydrolysis was determined by measuring the decrease in FRET as a function of time (Figure 2.4B). As shown in Figure 2.4B and quantified in Table 2, mutation of Rac1 Cys18 to Ser or Asp did not significantly affect the GAP-mediated rate of GTP hydrolysis. These data further suggest that oxidation of Rac1 is unlikely to alter down-regulation by GAPs.

4. NMR analyses for Rac1S-SG and the sulfonic acid oxidation mimetic Rac1 C18D

Our data indicate that Rac1 C18 oxidation likely activates Rac1 in cells by promoting guanine nucleotide exchange, similar to the action of GEFs. To examine how oxidation of Rac1 alters guanine nucleotide binding, we employed NMR spectroscopy to investigate site specific perturbations in Rac1 upon mutation and oxidation. 2D $^{1}H/^{15}N$ HSQC experiments were conducted on wtRac1, Rac1 C18D, and Rac1 S-SG. Figure 2.5 shows a ^{1}H-^{15}N 2D HSQC overlay for wtRac1 and either Rac1 C18D (A) or Rac1 S-SG (C). Approximately 10% of the resonances broadened for both Rac1 C18D and Rac1 S-SG; several resonances also displayed changes in chemical shifts for Rac1 C18D (5%) and Rac1 S-SG (2%). Notably, the Asp mutation generated more perturbations than the slightly larger glutathione modification. Likely this disparity was observed because Rac1 S-SG is not fully glutathiolated; however, it is possible that the two modifications uniquely affect Rac1. These alterations were primarily in the switch I and II regions as

well as, interestingly, the insert region; these data indicate that residues in these regions were altered in response to the aspartate mutation and glutathiolation (illustrated in the ribbon model in Figure 2.5B and 2.5D for Rac1 C18D and Rac1 S-GS, respectively).

5. The sulfonic acid mimetic Rac1 C18D, but not wtRac1 and the redox insensitive C18S variant, is hyperactivated in HEK-293T cells

To verify that Rac1 retains effector binding and to validate the above observations in cells by demonstrating that oxidative modification at Rac1 Cys18 leads to activation by enhancing nucleotide exchange, we examined whether Rac1 was activated as determined through RBD pull-down assays. We expressed wtRac1, the redox insensitive Rac1 C18S variant, and the sulfonic acid mimetic Rac1 C18D in HEK-293T cells. WtRac1 and Rac1 C18S showed similar activation levels. However, Rac1 C18D showed significantly higher levels of activation, as assessed by comparing the relative levels of Rac1-GTP capable of binding PAK-RBD. These results are consistent with our *in vitro* findings that demonstrate enhanced nucleotide dissociation for Rac1 S-SG and Rac1 C18D, but not wtRac1 or Rac1 C18S. As we have previously published that activated Rac1 is enhanced in HeLa cells the presence of H_2O_2 compared with untreated cells, our Rac1 C18S and C18D data herein further support the hypothesis that direct oxidative modification of Rac1 Cys18 can modulate the activation state of Rac1.

E. Discussion and Conclusions

Among its many vital functions , Rac1 plays a key role in regulating redox enzymes, including the RNS- and ROS-modulating proteins NOS and NOX (*4, 5, 208-213*). Moreover, recent data suggests that Rac1 interacts with SOD1, an antioxidant enzyme that converts superoxide into hydrogen peroxide (*6*). In addition to its role in regulating redox enzymes, Rac1 activity can be modulated by reacting with RNS and ROS (*13, 202, 225*). We have previously shown that treatment of Rac1 with either H_2O_2 or reagents capable of thiyl radical formation at Cys18 enhances GDP dissociation of Rac1 and facilitates exchange of GDP for GTP *in vitro* (*13, 225*). Further, we have demonstrated that H_2O_2 addition to HeLa cells leads to Rac1 activation (*202*). As Cys18 is located in the p-loop and forms multiple interactions with the bound nucleotide, we hypothesized that Cys18 in Rac1 has a lowered pK_a and, thus, is sensitive to oxidative modification. Oxidative modification of this residue in Rac1, in turn, likely perturbs interactions with the bound phosphate, enhancing the rate of nucleotide dissociation and promoting exchange of GDP to GTP (*228*). Thus, we assessed whether the pK_a of Rac1 Cys18 is altered and characterized whether oxidative modification of Cys18 alters Rac1 activity *in vitro*. In addition, we show that a sulfonic acid oxidation mimetic at Rac1 Cys18 (Rac1 C18D) is hyperactivated in HEK-293T cells compared with wtRac1 and a redox-insensitive variant (Rac1 C18S).

To determine whether Rac1 C18 possesses an altered pK_a, we employed a fluorescence based assay using a compound (ABD-f) that preferentially reacts with the cysteine thiolate. Results from these studies indicate that the Rac1 Cys18 pK_a is approximately 6.8, which is ~ full 2 pH units lower than a typical free cysteine thiol pK_a

and that Rac1 Cys18 is likely partially oxidized under physiological conditions. Consistent with a lowered pK_a at Rac1 Cys18, treatment of Rac1 with oxidized glutathione caused site-selectively modification of this residue at a physiological pH, but not 4 other cysteine residues in Rac1 (1-188 C178S). Moreover, glutathiolation of Rac1 Cys18 significantly enhanced the GDP dissociation rate (200-fold) compared with wtRac1 and the redox-insensitive variant Rac1 C18S. In addition, we generated a sulfonic acid oxidation mimetic at Rac1 Cys18 (Rac1 C18D). Similar to results obtained from Rac1 SS-SG oxidation, we find that Rac1 C18D exhibits an approximately 200-fold enhanced GDP dissociation rate over wtRac1 and Rac1 C18S. Notably, the thermal stability as well as intrinsic and GAP-mediated GTP hydrolysis kinetics were not altered upon mutation of Cys18 to either a Ser or Asp. As these studies were conducted in vitro, we also employed cell-based analyses to measure the level of activated wtRac1, Rac1 C18S, and Rac1 C18D. While Rac1 C18S showed similar Rac-GTP levels to wtRac1 when expressed in HEK-293T cells, hyperactivation was observed for Rac1 C18D. These results suggest that oxidation of Rac1 can lead to activation, through enhancement in the rate of nucleotide exchange.

We also investigated the regions of Rac1 affected by oxidation using NMR spectroscopy. As we would expect, Rac1 glutathiolation perturbs residues in the switch I and II regions; these regions are sensitive to the bound nucleotide and change conformation between the GTP- and GDP-bound forms of the protein. What we found surprising was that the insert region is also perturbed by oxidation at Cys18. The insert region is unique to Rho GTPases, and it is involved in certain effector interactions. These results suggest that binding of effectors that require the insert to Rac1 may be

sensitive to Rac1 oxidation. Given these findings, it would be interesting to perform a series of experiments that are directed toward discerning which Rac1 effector interactions are perturbed by oxidation. Clearly Rac1 retains binding to the RBD domain because this was used for the pull-down experiments in our cell-based studies, but perhaps oxidation perturbs binding to p67, for example, as studies indicate that the Rac1 insert region may be involved in this interaction.

These observations open up an entirely new line of investigations regarding Rac1 oxidation and redox regulation. First, while glutathiolation is a well-known post-translational oxidative modification, in cells, Rac1 is proximal to a variety of oxidants. Perhaps Rac1 incurs specific oxidative modifications depending on its microenvironment (e.g., an NO modification from NOS), which yield unique physiological consequences. We used aspartic acid as a mimetic for sulfonic acid because it is adds a comparable molecular weight and, more importantly, a negatively charged group. To better gauge the range of potential modifications, asparagine might be a nice mimetic for an NO or sulfonic acid modification because it would simulate the additional molecular weight and polarity. Further, we could modify the method set out herein that takes advantage of the lowered Cys18 pKa to selectively modify this residue with additional nitroso or oxidative groups. Second, because oxidation of Rac1 perturbs residues in the switch I and II as well as the insert regions, which are known to play a role in effector recognition, oxidization of Rac1 may alter interactions with specific effectors. Third, the Rac1 variants used and discussed herein may provide the tools necessary to discern a direct link between Rac1 and the various redox-modulatory events that it regulates in cells. Discriminating the in cell effects from the various potential oxidative modifications that Rac1 may incur would

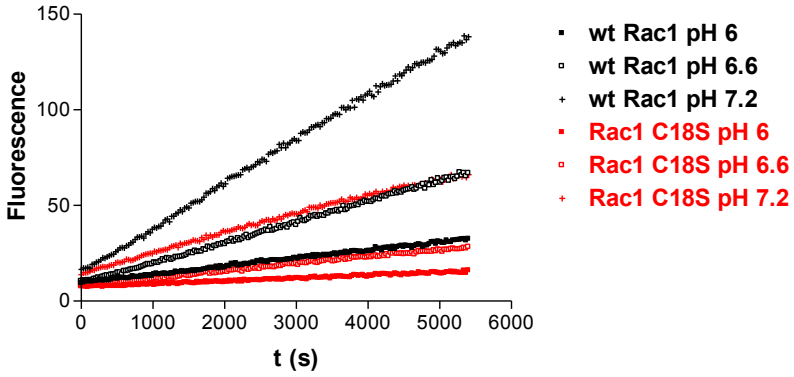
be especially insightful because such events in cells are typically difficult to distinguish, as oxidants have incredibly short half-lives and such events may be too transient for current detection methods.

In summary, our *in vitro* and cell-based experiments suggest that direct oxidative modification of Rac1 can alter Rac1 activity by enhancing the rate of nucleotide exchange and supports a new layer of regulation for activation of this protein through redox modification. Our observations are particularly interesting because Rac1 plays a critical role in controlling redox homeostasis and signaling in cells. Thus, redox regulation of Rac1 may not only directly modulate Rac1 activity but provide a feedback mechanism for the regulating major ROS- and RNS-modulating enzymes in the cell, including NOS, NOX and SOD1.

Figure 2.1: Rac1 Cys18 has a lower pK_a. (A) Thiol reactivity for wtRac1 showing Rac1 Cys18 reaction rate as a function of pH. (B) ABD-F reactivity data at three different pH values for wtRac1 and Rac1 C18S.

Figure 2.1

A.


150
100
50
0
Fluorescence
0
1000
2000
3000
4000
5000
6000
t (s)
wt Rac1 pH 6
wt Rac1 pH 6.6
wt Rac1 pH 7.2
Rac1 C18S pH 6
Rac1 C18S pH 6.6
Rac1 C18S pH 7.2

B.

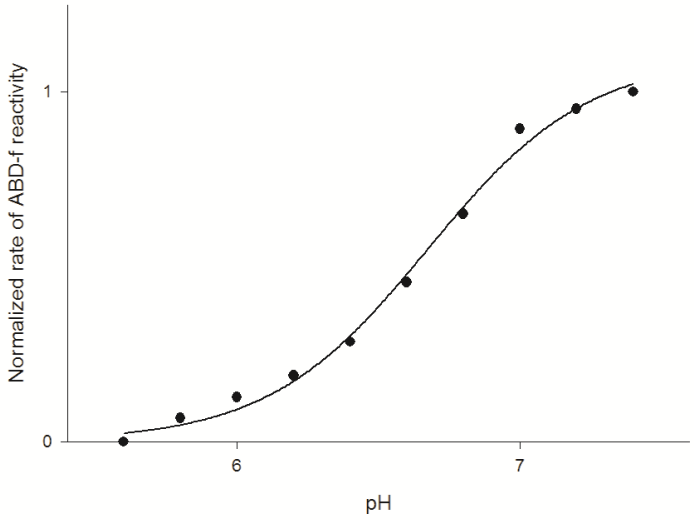

Normalized rate of ABD-f reactivity
1
0
6
7
pH

Figure 2.2: Rac1 Cys18 is selectively oxidized by GSSG. Full-length ESI-MS of the redox-insensitive control Rac1 C18S untreated (A) and treated with GSSG (B) as well as wtRac1 untreated (C) and treated with GSSG (D). Rac1 was also enriched with GSSG through an iterative process, which is shown in the inset in (D).

Figure 2.2

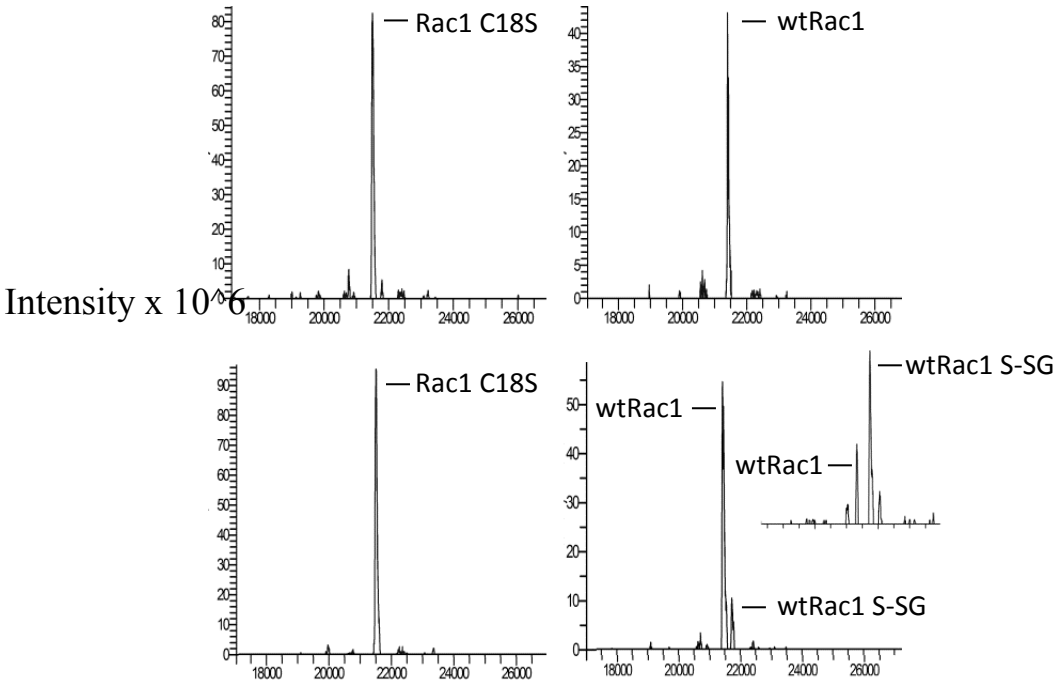

Intensity x 10^6
— Rac1 C18S
— wtRac1
— Rac1 C18S
wtRac1 —
wtRac1 —
—wtRac1 S-SG
— wtRac1 S-SG
Mass (Da)

Figure 2.3: Oxidation of Rac1 Cys18 alters guanine nucleotide binding and promotes intrinsic GDP dissociation. Intrinsic and Tiam1 (GEF)-mediated GDP dissociation kinetics for wtRac1 (black), Rac1 C18S (blue), Rac1-GS (green), and Rac1 C18D (red) at pH 7.5. Rac1 was preloaded with mant-GDP, excess unlabeled GDP, and the rate of rate of GDP dissociation determined by monitoring the loss of mant-GDP mant-GDP as a function of time at pH 7.5. The data were fit to a single exponential and standard error were determined using Prism 3.03 (n=3).

Figure 2.3

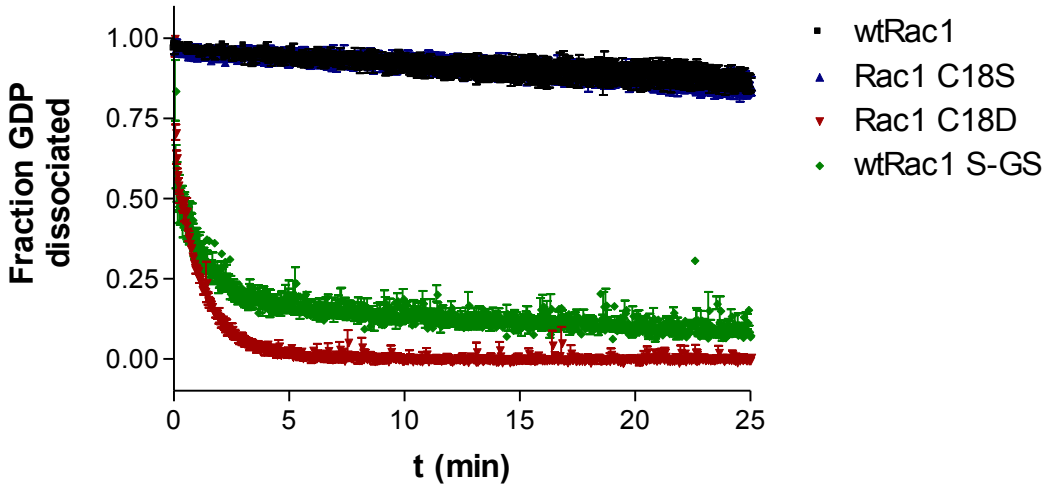

Fraction GDP dissociated
1.00
0.75
0.50
0.25
0.00
0
5
10
15
20
25
t (min)
wtRac1
Rac1 C18S
Rac1 C18D
wtRac1 S-GS

Table 1: Intrinsic and GEF-mediated rates of GDP dissociation for wtRac1, Rac1 C18S, Rac1 C18D, and Rac1S-SG

Table 1

Rac1 variant	k_{off} (s^{-1}) Intrinsic	Fold change from wtRac1	k_{off} (s^{-1}) with Tiam1	Fold change from wtRac1 with Tiam1
wt	$0.7 \pm 0.0 \times 10^{-4}$	n/a	$5.0 \pm 0.0 \times 10^{-4}$	n/a
C18S	$0.8 \pm 0.0 \times 10^{-4}$	1.1	$4.3 \pm 0.0 \times 10^{-4}$	0.9
C18D	$157.9 \pm 1.4 \times 10^{-4}$	223.3	$114.7 \pm 0.6 \times 10^{-4}$	22.9
Cys18S-SG	$167.5 \pm 25.1 \times 10^{-4}$	236.9	n/d	n/d

Figure 2.4: Mutation of Rac1 Cys18 does not perturb protein stability or intrinsic and GAP-mediated GTP hydrolysis. (A) Thermal stability of wtRac1 (black), Rac1 C18S (blue), and Rac1 C18D (red) measured by ABD-f reactivity as a function of temperature (30-70 °C) at pH 7. (B) Rates of intrinsic and GAP-mediated single-turnover hydrolysis for wtRac1 (black), Rac1 C18S (blue), Rac1-GS (green), and Rac1 C18D (red) at pH 7.4; n=3.

Figure 2.3 A

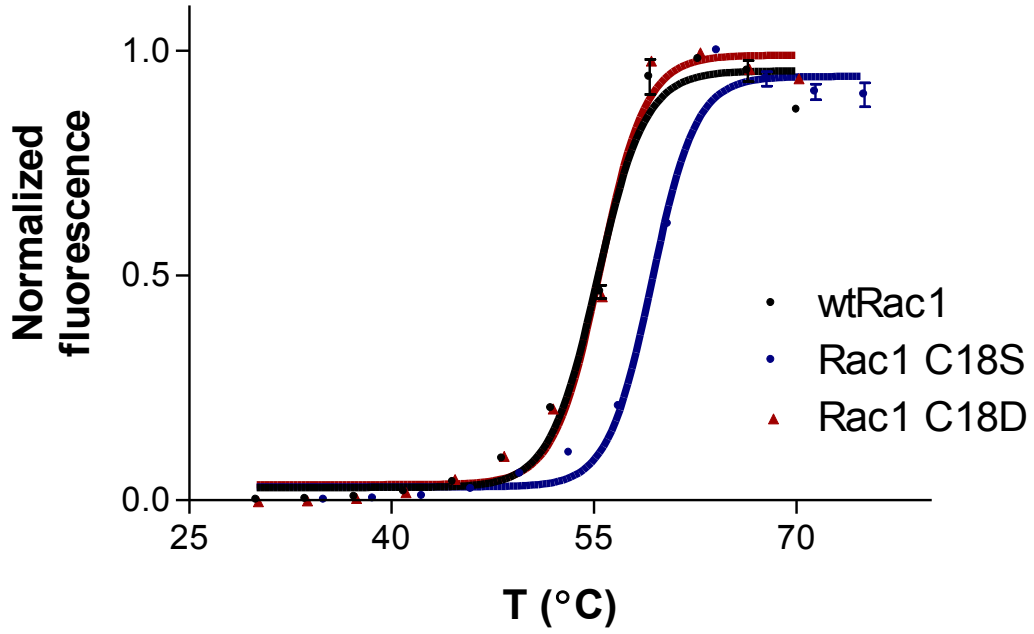

Normalized fluorescence
1.0
0.5
0.0
25
40
55
70
T (°C)
wtRac1
Rac1 C18S
Rac1 C18D

Figure 2.4 B

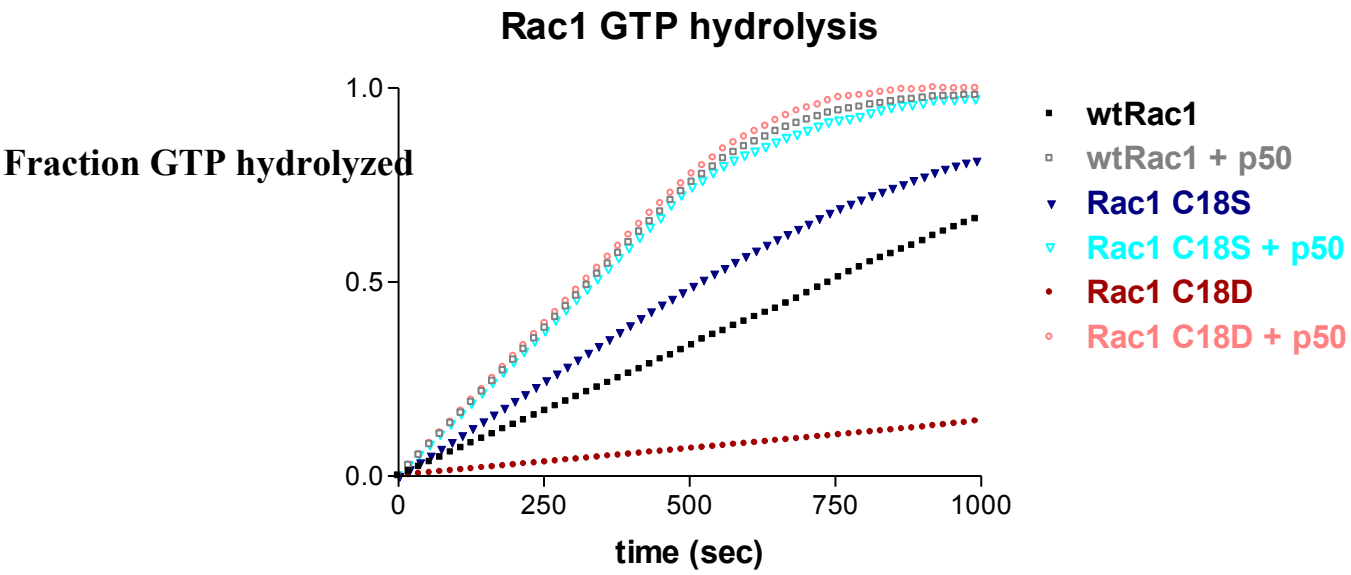

Rac1 GTP hydrolysis
Fraction GTP hydrolyzed
1.0
0.5
0.0
0
250
500
750
1000
time (sec)
wtRac1
wtRac1 + p50
Rac1 C18S
Rac1 C18S + p50
Rac1 C18D
Rac1 C18D + p50

Table 2: Intrinsic and GAP-mediated GTP-hydrolysis rates for wtRac1, Rac1 C18S, and Rac1 C18D

Table 2

Rac1 variant	Intrinsic GTP hydrolysis rate (s^{-1})	Fold change from wtRac1	p50 RhoGAP hydrolysis rate (s^{-1})	Fold change from wtRac1 + Tiam1
wt	$1.3 \pm 0.0 \times 10^{-3}$	n/a	$4.8 \pm 0.2 \times 10^{-3}$	
C18D	$0.2 \pm 0.0 \times 10^{-3}$	8.1	$4.8 \pm 0.3 \times 10^{-3}$	1
C18S	$1.4 \pm 0.1 \times 10^{-3}$	1.1	$5.1 \pm 0.4 \times 10^{-3}$	1.1

Figure 2.5: Rac1 regions affected by oxidation. (A) ^{1}H-^{15}N 2D HSQC NMR spectral overlay of wtRac1 (black) and Rac1 C18D (red). (B) Ribbon diagram of the Rac1-GDP (PDB Code 1MH1); highlighted residues show altered intensity or perturbed chemical shift upon mutation to the oxidation mimetic, C18D. The residues were based on previous assignments (BMRB Entry 5511). Darker green indicates line broadening (in the Rac1 C18D spectrum, the indicated residues have 50% of the linewidth compared with wtRac1), and yellow indicates that the signal disappears entirely (11% of the total number of residues detected); cyan indicates a chemical shift at least greater than one linewidth (5% of the total number of residues detected); and black indicates no information (residues that are undetected or unassigned). The Mg^{2+} ligand is indicated as a red sphere, the nucleotide is illustrated with sticks, and the redox-sensitive Cys18 is purple. (C) HSQC spectra of wtRac1 (black) and Rac1-GS (red). The residues were identified based on previous assignments. (D) Ribbon model of the Rac1 structure; residues are highlighted that decrease in intensity or produce a perturbed chemical shift upon oxidation with glutathione. The colors indicate the same as in (B); 9% percent of the total residues detected were broadened, and 2% of the detected residues underwent a chemical shift.

Figure 2.5 A

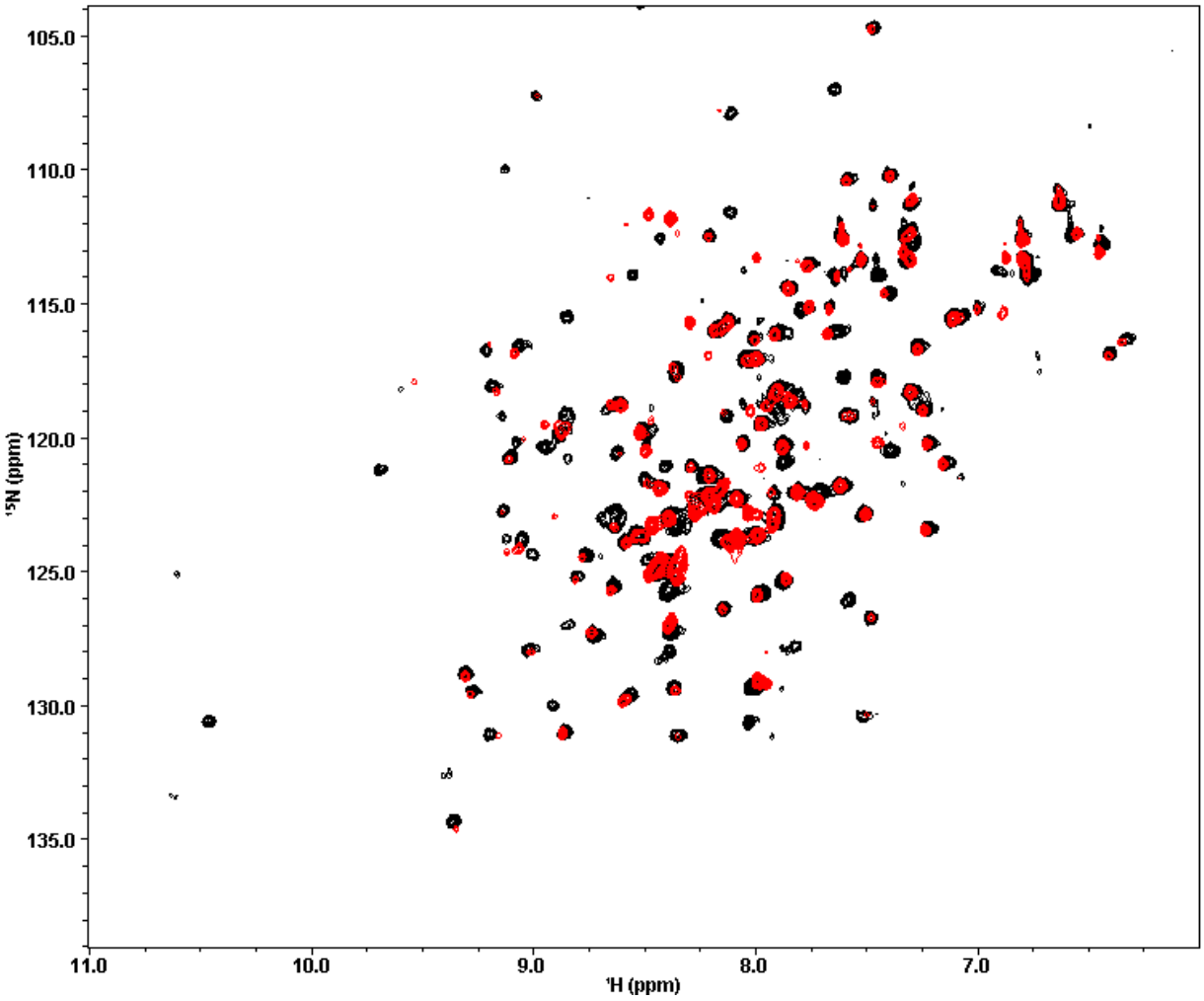

105.0
110.0
115.0
120.0
125.0
130.0
135.0
^{15}N (ppm)
11.0
10.0
9.0
8.0
7.0
^{1}H (ppm)

Figure 2.5 B

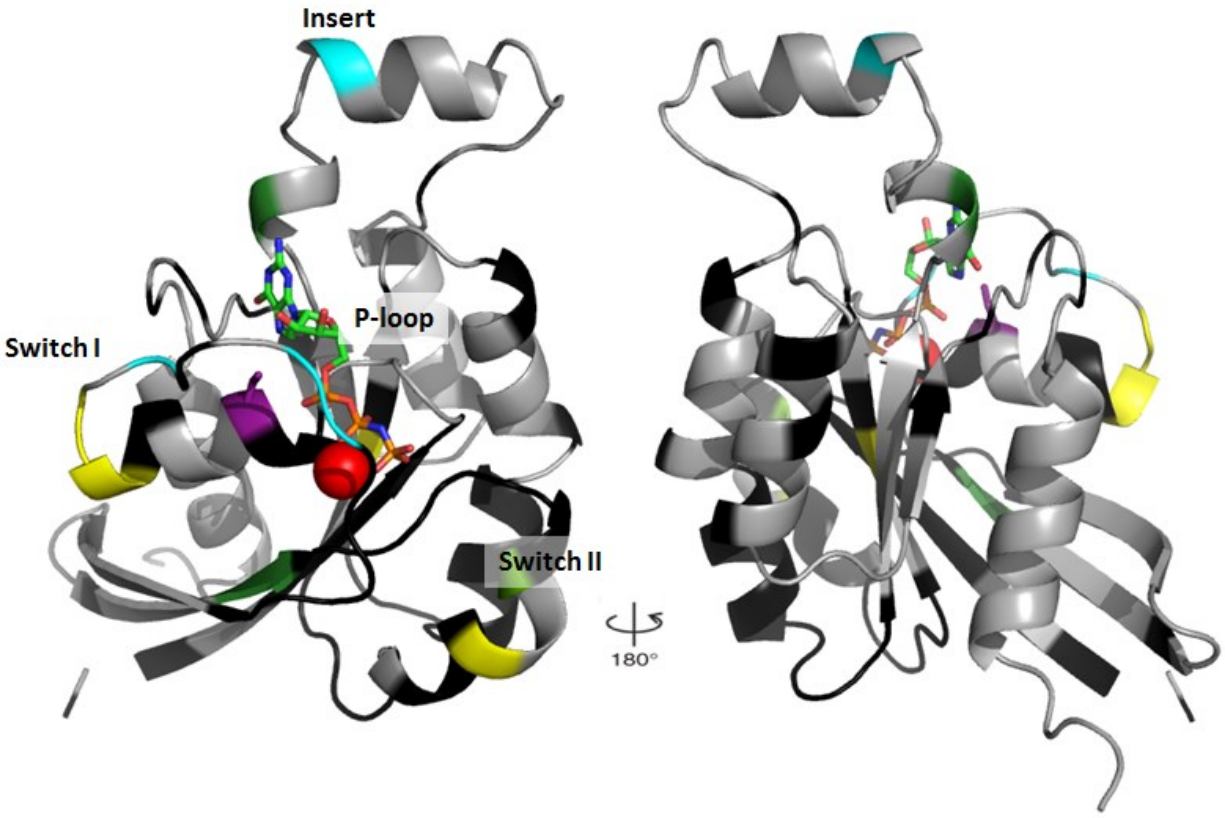


Figure 2.5 C

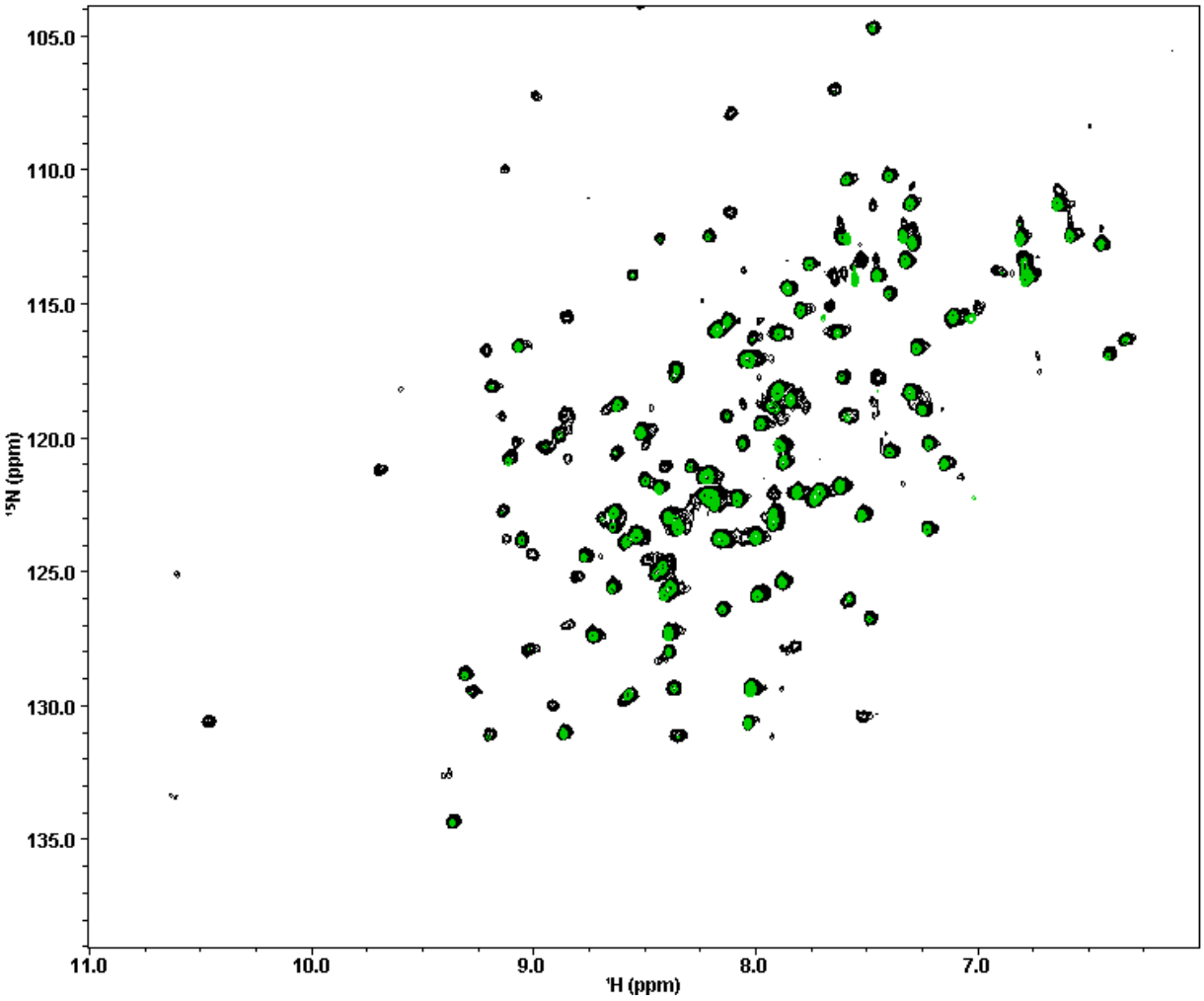

105.0
110.0
115.0
120.0
125.0
130.0
135.0
^{15}N (ppm)
11.0
10.0
9.0
8.0
7.0
^{1}H (ppm)

Figure 2.5 D

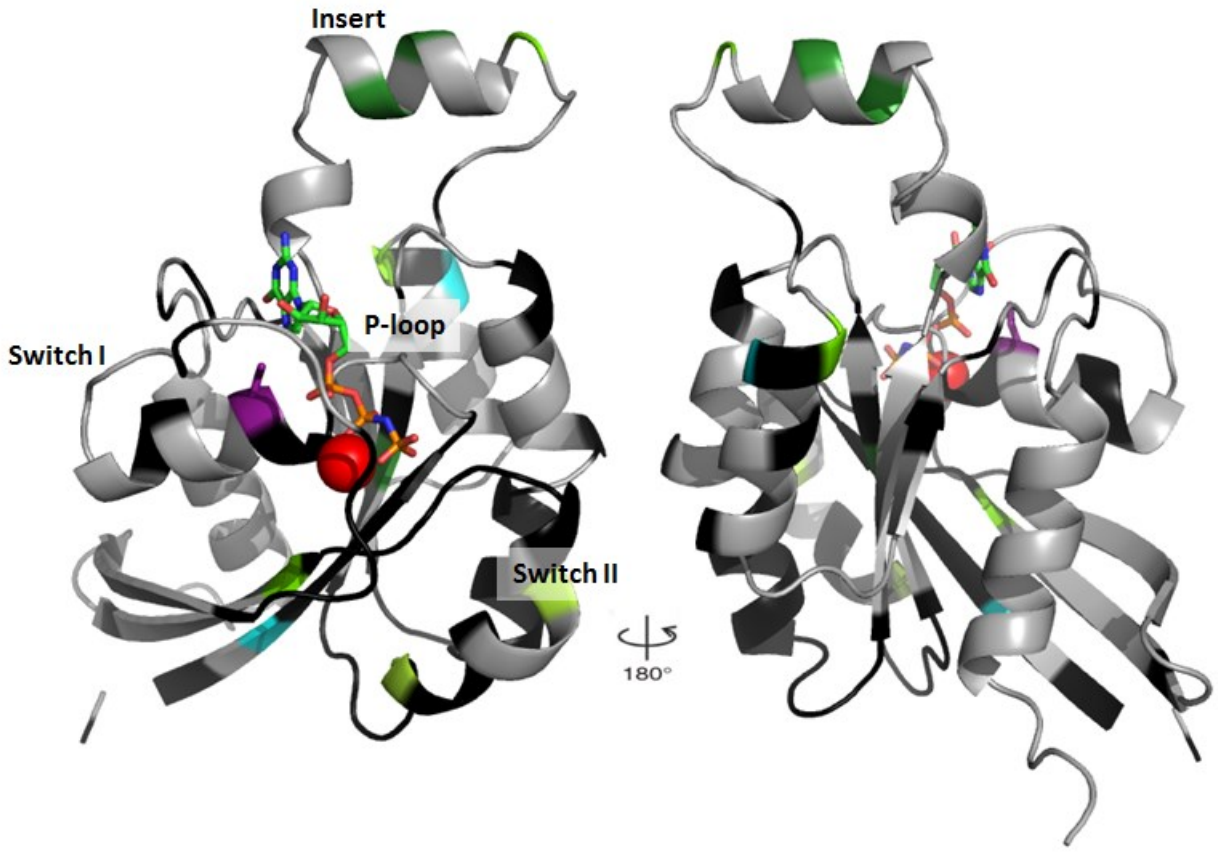


Table 3: Line-broadening and chemical shifts for Rac1 C18D and Rac1 S-SG. Only the resonances with greater than or equal to 50% line-broadening are plotted as the percent linewidth compared with unmodified wtRac1. Only the resonances with greater than or equal to 1 linewidth are plotted as the ppm change compared with unmodified wtRac1; the total linewidth for the indicated residue is plotted in parentheses; nc means no change was observed.

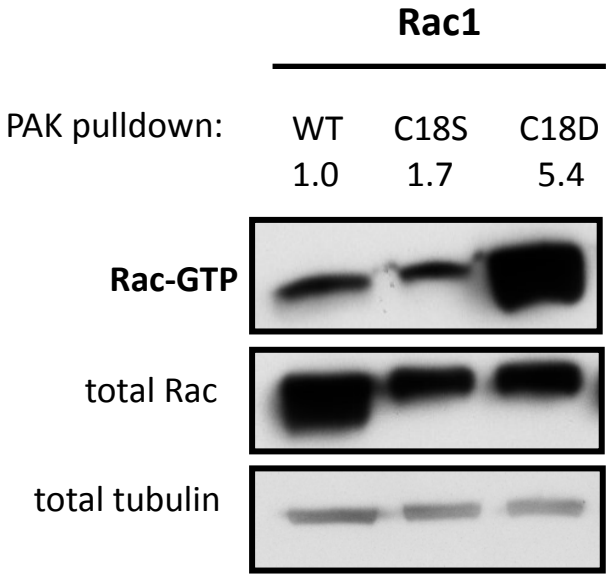
Table 3

	Line broadening (^{1}H ppm, % linewidth compared with unmodified wt Rac1)		Chemical shift change (^{1}H/^{15}N ppm shift [total ^{1}H/^{15}N linewidth ppm])	
	Rac1 C18D	Rac1 S-GS	Rac1 C18D	Rac1 S-SG
5K				0.19/0.62 (0.10/0.07)
24T	0			
25T	0			
26N	0			
28F			0.05/0.5491 (0.04/04)	
32Y			0.10/0.29 (0.04/.04)	
33I			0.13/0.18 (0.13/0.06)	
55L	49.2			
56W	49	43.3		
64Y	45.3			
65D		37.9		
67L	0			
71S		31.5		
80I	0	50.1		
89S			nc/0.90 (nc/0.04)	

118D	50.6			
124D	0		0.10/0.10 (0.04/0.04)	
125T		50.8		
127E		47.5		
131E		46.9		
139Y		46.5		
141Q	40.7		0.05/0.40 (0.10/0.28)	
158S	0			
185R		0		

Figure 2.6: A Rac1 mutant oxidation mimetic (Rac1 C18D) displays a constitutively high level of the active, GTP-bound Rac1 in cells. WtRac1, Rac1 C18S (redox-insensitive) and Rac1 C18D (sulfonic acid mimetic) were expressed in HEK-293T cells. Standard PAK pulldown assays were then used to assess the levels of active, GTP-bound Rac1, whereby active Rac1 pulled down with PAK-PBD coupled to agarose beads was detected by immunoblotting for the HA-epitope tag. A representative pulldown (n=6) is shown. This data was collected by Molly DeCristo in Adrienne Cox's laboratory.

Figure 2.5


Rac1
PAK pulldown:
WT
C18S
C18D
1.0
1.7
5.4
Rac-GTP
total Rac
total tubulin

Works Cited

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III. Is SOD1 a novel Rac1 effector?

A. Overview

Rac1 is a GTPase in the Rho subfamily of the Ras superfamily. It plays a role in multiple critical cell processes, including cell morphology and motility, cell cycle regulation, gene expression, as well as redox homeostasis and signaling. Thus, is unsurprising that when Rac1 is mutated or dysregulated, it can produce a host of pathophysiological conditions, such as cancer, cardiovascular disease, and neurological disorders. As Rac1 is heavily involved in regulating redox homeostasis and signaling, it binds to and controls activation of redox-modulating enzymes, including Nox and NOS, which are major producers of ROS and RNS (respectively) in the cell. Further, a recent study demonstrated that Rac1 binds SOD1 in a nucleotide- and redox-dependent manner. SOD1 is considered an antioxidant enzyme in the cell because it dismutates the highly reactive superoxide to hydrogen peroxide. I show preliminary data that suggests SOD may binds Rac1 in a redox- and nucleotide-dependent manner. Further, because I have established that oxidation of Rac1 Cys18 alters its activation state and perturbs residues in and around the regions of the protein known to play a role in effector binding, I investigated the effect of Rac1 Cys18 redox modification on SOD1 binding. I demonstrate that redox modification at this site alters SOD1/Rac1 binding. However, both the nucleotide-dependence and Cys18 redox modification binding assays comprise a

certain level of ambiguity, as the binding curves indicate that either the SOD1 or Rac1 sample has greater than one predominant population. Thus, I further used NMR experiments to discern a binding event between Rac1-GDP and SOD1, which suggested that Rac1 and SOD1 bound with low affinity, as would be expected for Rac1-GDP and SOD1. Interestingly, the NMR-based experiments further showed that residues in and around the regions of Rac1 involved in effector binding were perturbed when SOD1 was added. Thus, I demonstrate that Rac1 binds SOD1; however, future experiments should be directed at clarifying this event and discerning the source of the ambiguity.

B. Introduction

Rac1 is a small GTPase in the Rho subclass of the Ras superfamily. As such, it plays a strong role in multiple fundamental signaling pathways, including pathways that control cell motility and morphology, cell cycle, gene expression, as well as redox homeostasis and signaling (*2*). Given that Rac1 regulates so many critical cell events, it is unsurprising that Rac1 deletion is embryonic lethal, and mutation or dysregulation of Rac1 can facilitate cancer, cardiovascular disease, and neurological disorders (*6-9, 39, 66, 114*). The paradigm for activation of small GTPases (including Rac1) is a nucleotide switch mechanism, whereby GDP-bound Rac1 populates an inactive conformation, but Rac1-GTP is the activated from of the protein (*1, 20*). Rac1-GTP must hydrolyze the γ phosphate to generate the inactivated form; however, this process in intrinsically slow, and requires GAPs to aid in catalysis (*20*). Further, small GTPases bind GDP and GTP with similar affinity, and because the in cell environment comprises a GTP:GDP ratio of at least 10:1, Rac1-GDP must release the bound nucleotide to facilitate the GTP-bound activated form (*19, 20, 27*). Nucleotide dissociation is also intrinsically too slow to

respond to cell signals; thus, GEFs are required to aid in nucleotide dissociation (*20, 27*). Notably, post-translational modifications have also been shown to regulate Rac1 activity in cells, such as phosphorylation, ubiquitination, SUMO-lation, and lipid modification (*32, 33, 36, 38*). Moreover, the Campbell lab has shown previously and I have shown herein that oxidants can stimulate GDP dissociation, which likely produces the activated form in cells. Thus, oxidants are likely an additional layer of regulation for Rac1 activation.

As a significant player in regulating redox homeostasis and signaling in cells, Rac1 is unique in its interaction with and activation of multiple enzymes that produce and/or modulate levels of ROS/RNS. Rac1 interacts with and directly stimulates Nox activity (*4*). It interacts with and regulates NOS activity, and one report suggests that it may directly stimulate NOS activation (*208-213*). Additionally, a recent study has shown that Rac1 directly interacts with SOD1 in a redox- and nucleotide-dependent manner (*6*). SOD1 is considered an antioxidant enzyme in the cell because is dismutates the highly reactive free radical superoxide to a less reactive non-radical oxidant, H_2O_2 (*195*). This same study demonstrated that SOD1-ALS variants bound Rac1 and abrogated the nucleotide and redox dependence of this interaction (*6*). Harraz, et al. further provided evidence that SOD1-ALS mutant/Rac1-GTP binding stabilized activated Rac1 and corresponded to Nox-dependent superoxide production (*6*). Thus, they proposed that SOD1 bound Rac1-GTP under reducing conditions and facilitated Rac1/Nox binding as well as Nox activation (*6*). Further, their model asserted that SOD1-ALS variants bound Rac1-GTP regardless of redox status, which facilitates Nox-mediated ROS production absent redox-based feedback (*6*). Interestingly, Rac1 has been connected to ALS

independent of this SOD1 interaction (*178-182*). It has been shown that familial ALS (fALS) is derived from mutations both in SOD1 and a Rac1 effector, alsin (*178, 179, 182*). SOD1 and alsin modulate a redox-mediated proinflammatory signaling pathway through Rac1, PI3K, Akt, and Nox (*182*). Thus, I was interested in exploring this interaction. The Rac1/SOD1 study was predominantly cell-based, and I sought to examine the SOD1/Rac1 interaction in vitro using biochemical and biophysical methodologies.

Below, I present preliminary data that suggest Rac1 may interact with SOD1 in a redox- and nucleotide-dependent manner. Further, the NMR-based studies demonstrate that residues in and around the Rac1 switch I and II as well as insert regions are perturbed upon SOD1 binding. This finding is particularly interesting because, while the switch I and II regions are typically involved in Rac/effector interactions, the insert region likely only contributes to interactions with certain effectors (*101, 205, 206*). Most notably, studies show that the insert regions may play a role in the Rac1/Nox interaction, which is especially intriguing in light of Harraz, et al.'s model that depicts interplay between the Rac1/Nox and Rac1/SOD interactions with implications for disruption by SOD1-ALS variants (*205, 206*).

C. Methods and Materials

1. Protein expression and purification

Human wtRac1 (1-188, C178S) and the Cys18 variants were expressed in the pET 15b vector system (EMD Millipore). Escherichia coli BL21 (DE3) RIPL cells (Strategene) were transformed with the Rac1 expression system. The *E. coli* cells were grown at 37°C until 0.6 $O.D._{600}$; the cells were then treated with 1 mM isopropyl β-D-1-

thiogalactopyranoside (IPTG). After 4 more hours at 37°C, the cells were centrifuged, and the cell pellet was lysed through sonication in 50 mM KH_2PO_4 pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 10 μM GDP, and 5 mM β-mercaptoethanol (BME). The Rac1 construct contains a HIS-tag and was purified using a Ni-NTA column (Qiagen) to >95% purity by SDS-PAGE. Rac1 was stored in 50% glycerol at -20°C. SOD was purified as previously described (*236*).

2. FRET-based binding assay

A previously described assay was modified as follows (*234*). Rac1 was pre-loaded with mant-GDP. Rac1 Trp56 is sufficiently proximal to the bound nucleotide for FRET to occur, and I demonstrate that Trp56 is perturbed upon Rac1/SOD1 binding. Thus, I excited Rac1 Trp56 (280 nm) and measured the decrease in FRET-mediated fluorescence emission from the bound nucleotide (435 nm) as a function of [SOD1] to produce a binding curve for Rac1 and SOD1. Because SOD1 is also excited at 280 nm and produces a broad emission peak at 365 nm that extends to 435 nm, I measured the minor SOD1 emission signal at 435 nm and subtracted this data from the FRET signal. I then fit the data to an exponential function (Prism 3.03). The binding assays were performed at room temperature in 20 mM Tris pH 7.5, 150 mM NaCl, and 1 mM DTT. SOD1 and Rac1 were incubated at room temperature for 30 min to equilibrate.

3. HSQC experiments

Rac1 was expressed and purified as described above except that the medium supplied to the E. coli cells during expression was ^{15}N-enriched M-9 minimal media. The ^{1}H-^{15}N HSQC experiments were performed using a Varian Inova 700 MHz spectrometer with a cryoprobe in 50 mM Tris Malate pH 6.8, 50 mM NaCl, 5 mM MgCl2, 50 μM

GDP, 0.1 mM DTPA, and 1 mM DTT (DTT was not added to the glutathiolated sample). The Rac1 concentration was approximately 200 μM, and the SOD1 concentration was varied from 20 μM – 1 mM.

D. Results

1. Nucleotide dependence

Harraz et al.'s study suggested that Rac1-GTP bound SOD1 under reducing conditions, but SOD1 bound only Rac1-GDP under oxidizing conditions (*6*). Nucleotide-dependent binding is expected for Rac1/effector interactions because typically effectors recognize and bind Rac1 in its GTP-bound conformation. Thus, I first tested the Rac1/SOD interaction under reducing conditions using the GTP- and GDP-bound forms of the protein. I modified a FRET-based method used in the field to detect Rac1/SOD1 binding (*234*). First, Rac1 was preloaded with a fluorescently labeled (mant) nucleotide (GDP or the non-hydrolyzing GTP analog GMPPCP). Then a Trp residue that is proximal to the bound nucleotide (Trp56) was excited at 280 nm; the Trp residue is sufficiently proximal to the nucleotide for FRET to occur, and emission was detected for the nucleotide-bound mant fluorophore (435 nm). However, as verified by our NMR results (below), SOD1 binding perturbs this Trp residue, and when SOD1 binds, the FRET signal is decreased. Thus, I measured SOD binding as a function of the decrease in FRET from the Rac1 Trp56 to the mant-labeled nucleotide. As shown in Figure 3.1, the binding curves for Rac1-GDP/SOD1 and Rac1-GMPPCP/SOD1 are distinct and indicate a nucleotide-mediated binding event, which would be expected for a Rac1/effector interaction. However, an inhomogeneity is apparent in the Rac1/GMPPCP binding curve, which may be generated by SOD1, Rac1, or the assay. Future experiments should be

directed towards discerning either the population of Rac1 or SOD1 responsible for this differential apparent binding or whether the apparent inhomogeneity is generated from an artifact of the assay.

2. Redox dependence

Harraz, et al. also observed that the SOD1/Rac1 interaction is redox-dependent (*6*). As I have demonstrated above that Rac1 Cys can be oxidized at a physiological pH, I investigated the effect of Cys18 oxidation on the Rac1/SOD1 interaction. I explored SOD1 binding with GMPPCP-bound wtRac1, Rac1 C18S, and Rac1 C18D using the FRET-based assay described in the above section. wtRac1-GMPPCP and SOD1 produced the same binding curve that depicts two distinct binding events. Rac1 C18S-GMPPCP demonstrated binding that indicates a single, low-affinity binding event, which is similar to the binding curve generated for wtRac1-GDP and SOD1. Rac1 C18D-GMPPCP produced a dataset comprising a dramatic apparent change in FRET between the data for 0 M SOD and 300 nM followed by apparent linear, non-specific binding for 300 nM – 100 μM SOD. It is clear that perturbation of Cys18 alters the interaction between Rac1 and SOD1; however, the apparent ambiguity in the binding data limits the conclusions that can be drawn therefrom. These data would benefit from binding data using a different assay that could either validate the data and inhomogeneity or discern an artifact in the FRET-based assay used herein.

3. Key regions in Rac1 are perturbed upon SOD1 binding

To explore the specific regions of Rac1 affected by SOD1 binding, I performed a series of HSQC experiments using ^{15}N-labeled Rac1 with varying levels of SOD. Unsurprisingly, residues in the Rac1 switch I and II regions were perturbed by adding

SOD1. Line-broadening was primarily observed likely because Rac1 interacts with SOD with a low micromolar binding affinity and such an interaction is typically within the intermediate exchange regime, which often produces line-broadened residues. It is possible, though, that the Cu molecule in SOD generates paramagnetic relaxation at the Rac1 residues proximal to this site. A paramagnetic relaxation event would likely affect residues on a particular portion of Rac1, and the line-broadened residues are dispersed. However, many residues proximal to the affected residues have not been assigned and may in fact undergo such relaxation. The perturbed switch I and II regions are sensitive to changes in the bound nucleotide and are involved in the interaction between Rac1 and various effectors (*101*). It is particularly interesting that residues adjacent to the insert region were also perturbed, as studies have suggested that the Rac1 insert region is involved in certain effector binding interactions, such as the Rac1/Nox interaction (*205, 206*).

E. Discussion and Conclusions

Rac1 is a ubiquitously expressed GTPase with a strong role in cell motility and morphology, cell cycle, gene expression, as well as redox homoeostasis and signaling (*2, 11*). Rac1 deletion is embryonic lethal, and mutation or dysregulation of Rac1 can facilitate such pathophysiological conditions as cancer, cardiovascular disease, and neurological disorders (*6-9, 39, 66, 114*). Rac1 activation is controlled through a nucleotide switch mechanism, whereby Rac1 bound to GDP populates an inactive conformation, and Rac1-GTP is the activated form (*1, 20*). The predominant paradigm in the field is that GEFs facilitate the activated form through promoting nucleotide release because Rac1 binds GDP and GTP with similar affinity, and the GTP:GDP ratio is

typically at least 10:1 in cells (*19, 20, 27*). Post-translational modifications are an additional mechanism for controlling Rac1 activity in cells, including phosphorylation, ubiquitination, SUMO-lation, and lipid modification (*32, 33, 36, 38*). I have shown above that oxidation at Rac1 Cys18 is likely an additional post-translational modification that can enhance the activated form of Rac1. It is interesting that Rac1 activation can be controlled through oxidation when Rac1 is known to interact with multiple enzymes that produce oxidants. Rac1 interacts with and directly stimulates Nox activity (*4*). It interacts with NOS and regulates its activity, and certain reports have suggested that Rac1 might also directly stimulate NOS activity (*208-213*). It was recently shown that Rac1 interacts with SOD1 in a redox- and nucleotide-dependent manner (*6*). This study was primarily cell-based, and I sought to investigate this interaction using biochemical and biophysical methodologies.

Harraz, et al. reported that the Rac1/SOD interaction was nucleotide-dependent, which is typical for Rac1/effector interactions (*6*). To test this finding biochemically, I used a FRET-based binding assay with SOD1 and Rac1-GDP or Rac1-GMPPCP. Our data showed a distinct binding curve for Rac1 bound to the different nucleotides. However, the Rac1-GMPPCP/SOD1 binding data are ambiguous and suggest an inhomogeneity in the Rac1 or SOD1 samples, or it suggests that the assay has produced an artifact. The SOD1 used for the experiments herein was derived from red blood cells (RBCs), and it comprises a distinct population of the SOD1 purified from the RBCs. It has been shown that SOD1 is post-translationally modified in RBCs, including phosphorylated and glutathiolated variants. The SOD1 used herein comprise a fraction of the SOD1 in RBCs that is separated using an anion exchange column and is typically

unmodified ("peak 1"). However, each SOD1 peak 1 sample is not analyzed for post-translation modifications, and it is possible that the SOD1 samples used for these experiments comprise a heterogeneous post-translationally modified population of SOD1.

On the other hand, the Rac1 samples may also be the source for inhomogeneity in the experiment. Harraz, et al. observed differential binding for Rac1 and SOD1 that was dependent on the redox status of Rac1 (*6*). Perhaps the reducing agent added was insufficient to produce a fully reduced Rac1 sample. If redox status of Rac1 does control its affinity for SOD1, an incompletely reduced Rac1 sample would produce an inhomogeneity with two species that bind SOD1 with different apparent affinities. I speculate that at least two Rac1 species are likely present in this sample (one reduced and one oxidized) for the following reasons. (i) The Rac1 oxidative modification experiments above suggest that oxidation at Cys18 may affect effector binding, and questions remain as to the various oxidation events and their impact on effector binding. (ii) Harraz, et al. observed that redox conditions affected the Rac1/SOD1 interaction, and the differential SOD1 binding curves for wtRac1, Rac1 C18S, and Rac1 C18D suggest that oxidative modification at Rac1 Cys18 affects SOD1 binding (*6*). Moreover, the NMR data indicates that SOD1 binding perturbs residues adjacent to the Rac1 insert region, which may play a role in effector recognition. Residues in this region were also perturbed in the Rac1 Cys18 oxidation experiments that are described above, which suggests that such modifications may affect effector binding. It is possible, however, that the FRET-based assay used to characterize this interaction has produced an artifact that appears as an inhomogeneity in the sample.

The first two experiments that might clarify the source of the ambiguity in the SOD1/Rac1 binding data would include a different method for detecting Rac1/SOD1 binding and a series of HSQC experiments with ^{15}N-labeled Rac1 C18S and C18D. A second method that produces similar apparent binding data for SOD and either wtRac1, Rac1 C18S, and C18D would at least exclude an artifact of the FRET-based assay as the basis for the ambiguity. An HSQC for ^{15}N-labeled Rac1 C18S and C18D may further validate differential binding between SOD and Rac1 with various oxidative modifications at Cys18.

In addition to validating and clarifying the Rac1/SOD1 binding data herein, it would be interesting to further investigate the specific regions of Rac1 and SOD1 involved in this interaction. The NMR data for wtRac1/SOD1 binding primarily depicts SOD1-mediated line broadening for certain Rac1 residues in the switch I and II as well as insert regions. However, line broadening may result from either a low-affinity binding interaction that produces intermediate exchange in the backbone amide protons or paramagnetic relaxation mediated by the Cu atom in the SOD1 active site. If the line broadening is generated through a low affinity interaction between Rac1 and SOD1, perhaps the above proposed SOD1 binding experiments with Rac1 C18S and C18D will aid in clarifying this interaction. However, if the Cu in SOD is the basis for the line broadening, then paramagnetic relaxation can provide distance information for the residues involved in the interaction between SOD1 Rac1 (*237-239*). SOD1 Zn/Zn has been previously generated and characterized; this SOD1 variant would be a nice control for discerning whether Cu is involved in the SOD1-mediated line-broadening for Rac1 (*240*). Moreover, if paramagnetic relaxation is the basis for the SOD1-mediated Rac1

line-broadening, complementary $^{1}H/^{15}N$ HSQC experiments using ^{15}N-labled Rac1 and SOD1 Zn/Zn may provide additional information on the residues in Rac1 perturbed upon SOD1 binding.

Using FRET-based experiments, I demonstrate that SOD1 binds Rac1 in an apparent nucleotide- and redox-dependent manner. However, the data suggests either an inhomogeneity in the sample or an artifact with the assay. I have suggested certain experiments that may aid in clarifying the Rac1/SOD1 interaction and/or discerning the source for the ambiguity. Further, I have described NMR experiments using ^{15}N-labeled Rac1 and SOD that suggest an effector-like interaction as well as future experiments that may generate additional information on the site of interaction between Rac1 and SOD1. Such data would be especially useful for future in vitro and cell-based studies if the information resulted in a mutation that disrupted the Rac1/SOD1 interaction.

Figure 3.1: Nucleotide-dependent Rac1/SOD1 binding. Rac1-GDP (red) and Rac1-GMPPCP (black, GTP analog) and SOD1 binding curves are shown. In contrast to Rac1-GDP, which binds SOD1 with a single apparent binding event (~40 μM), the SOD1/Rac1-GMPPCP binding curve shows two apparent binding events; one is low affinity (~40 μM), and the second is high affinity (~90 nM).

Figure 3.1

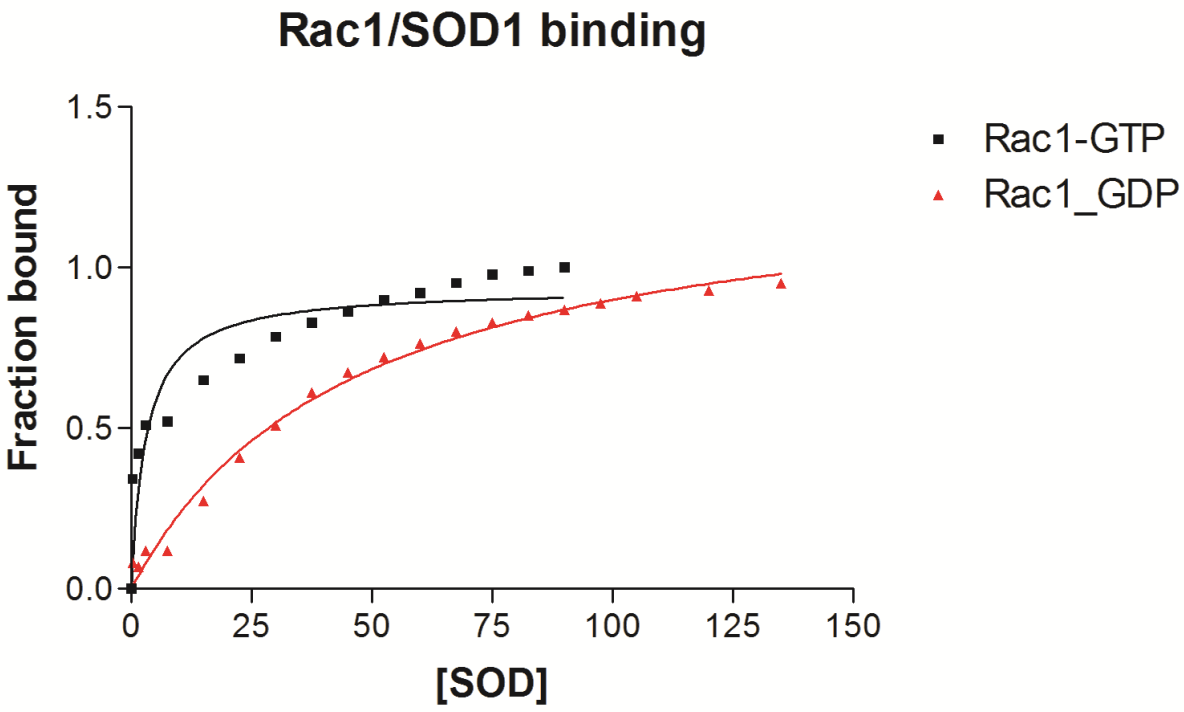

Rac1/SOD1 binding
Fraction bound
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
[SOD]
Rac1-GTP
Rac1_GDP

Figure 3.2: Rac1 Cys18 oxidation-dependent binding with SOD1. Rac1/SOD1 binding curves are shown for wtRac1 (black), the redox-insensitive variant Rac1 C18S (blue), and the sulfonic acid mimetic Rac1 C18D (red). As shown, the wtRac1/SOD1 binding curve shows the characteristic apparent dual binding events (~40 μM and 90 nM); Rac1 C18S produces an apparent single binding event (~40 μM); and Rac1 C18D produces a binding curve with a significant apparent increase in binding followed by a linear, non-specific binding event.

Figure 3.2

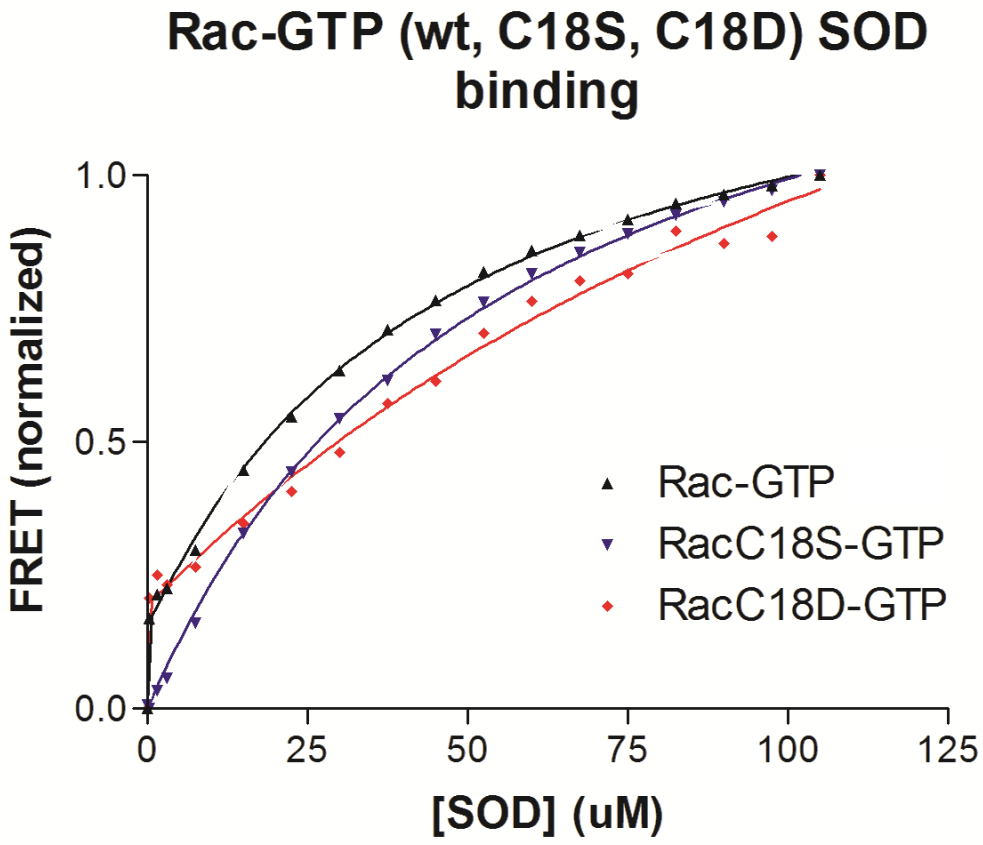
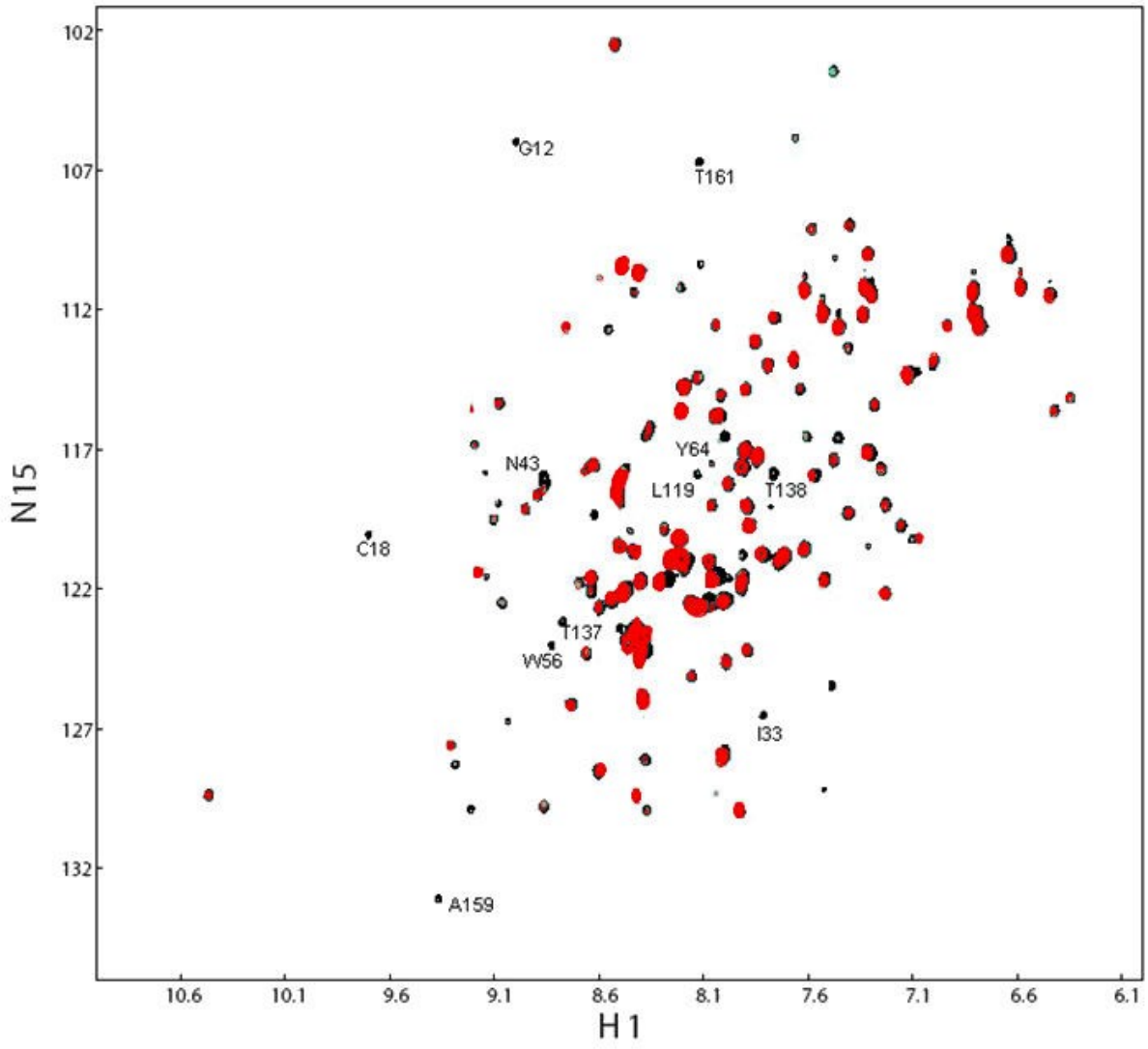

Rac-GTP (wt, C18S, C18D) SOD binding
FRET (normalized)
1.0
0.5
0.0
0
25
50
75
100
125
[SOD] (uM)
Rac-GTP
RacC18S-GTP
RacC18D-GTP

Figure 3.3: HSQC overlay of Rac1 with and without SOD1. (A) Line broadening was the primary alteration that we observed from the $^{1}H^{15}N$ HSQC experiment with Rac1-GDP and 5 molar excess SOD. The spectrum for Rac1 without SOD1 is in black, and the red overlay spectrum is Rac1 with SOD1. (B) The perturbed residues are mapped onto a ribbon model of Rac1 (PDB: 1MH1). The broadened residues are in yellow (peaks that disappear entirely) and blue (peaks that broaden by at least 50%, but do not disappear entirely). The residues with chemical shift perturbations are in pink. The unassigned residues are in grey, which are in the most mobile regions of the protein, the switch and effector regions. The Mg^{2+} and bound nucleotide are in red and brown, respectively.

Figure 3.3


102
107
112
117
122
127
132
N15
G12
T161
Y64
N43
L119
T138
C18
T137
W56
I33
A159
10.6
10.1
9.6
9.1
8.6
8.1
7.6
7.1
6.6
6.1
H1

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IV. Conclusions and Future Directions

Rac1 is a small GTPase in the Rho subclass of the Ras superfamily, which uses the prototypical nucleotide cycling mechanism to switch between its activated and inactivated forms (*2*). When Rac1 is GDP-bound, it is inactivated, and GTP-bound Rac1 populates the activated conformation. Rac1 binds GTP and GDP with similar affinities, and the GTP:GDP ratio is at least 10:1 in cells; thus, nucleotide release typically facilitates the activated form of the protein, and GTP hydrolysis inactivates Rac1 (*1, 19, 20, 27*). Intrinsically, these processes are too slow to respond to changes in the cell; thus, the regulatory proteins GAPs and GEFs facilitate GTP hydrolysis and GDP dissociation to produce the inactive and activated forms of the protein, respectively (*1, 19, 20, 27*). As with many small GTPases, Rac1 has a strong signaling function in cells. Rac1 is ubiquitously expressed and regulates cell motility, morphology, gene expression, and cell cycle (*2*). Rac1 is unique in the body of literature devoted to its role in regulating redox homeostasis and signaling. It directly interacts with Nox, NOS, and SOD, which are predominant redox-modulatory enzymes in the cell (*4, 6, 208-213*). Rac1 directly activates Nox and regulates the activity of NOS (*4, 208-213*). Given its ubiquitous nature and that it regulates multiple critical cell functions, it is unsurprising that Rac1 deletion is embryonic lethal, and when Rac1 is mutated or dysregulated, it can produce multiple pathophysiological conditions, including cancer, cardiovascular disease, and neurological disorders (*2, 6-9, 39, 66, 114*).

Because Rac1 is localized to multiple sites with high levels of ROS and RNS, I was interested in exploring the effect of Rac1 oxidation. The Campbell lab has previously demonstrated that radicals facilitate enhanced GDP dissociation in Rac1 (>500-fold), and H_2O_2 can enhances GDP dissociation by 10-fold (*13*). Further, the Campbell lab has shown that Rac1 oxidation is mediated through Cys18 (*13*). I have shown that mutation and oxidation of this site can dramatically perturb nucleotide binding; however, the level of perturbation depends upon the particular modification. Likely, in the cell the particular oxidative modifications will control the rate of Rac1 activation. In addition, the data herein demonstrate that oxidation likely perturbs residues in the switch I and II as well as the insert regions. As these regions are often involved in Rac1 effector recognition, oxidative modification may perturb certain Rac1/effector interactions (*101, 205, 206*). It would be interesting to perform a series of effector binding assays with various Rac1 effectors, with certain insights into any oxidant-mediated interaction perturbation, it may be possible to narrow down the pathways most affected by oxidation. The data both elucidate and characterize a novel post-translational modification for Rac1, but they also raise new and interesting questions. However, the site-selective oxidation method developed herein may also provide a new tool for answering certain questions in vitro such that cell-based analyses are more directed.

While I was investigating redox regulation of Rac1, the Engelhardt lab published a study showing that Rac1 interacted with SOD1 in a redox- and nucleotide-dependent manner and that SOD1 ALS variants interacted with Rac1 without a preference for redox and nucleotide status (*6*). The data in the Engelhardt study was primarily cell-based, and I was interested in quantitatively characterizing this interaction using biochemical and

biophysical techniques. Using a novel FRET-based technique, I produced preliminary data that suggests Rac1 and SOD may interact in a nucleotide- and redox-dependent manner; however, the data indicated a heterogeneity in the sample that I have been unable to identify (*234*). Nonetheless, using NMR, I did identify certain residues that were affected by SOD1 in and around the switch I, switch II, and insert regions, which are the same general regions involved in Rac1/Nox binding. As the Engelhardt study proposed a model wherein both SOD1 and Nox bound Rac1 under particular redox conditions, it would be interesting to test whether the presence of Nox and SOD1 with Rac1 alters its propensity to bind these proteins under such redox conditions (*6*). If we could identify the specific residues involved in the Rac1-SOD interaction, we may be able to construct a mutation that abrogates this interaction for cell-based studies. Though the data herein are not fully informative due to an unidentifiable heterogeneity in the sample, the results are intriguing and raise a host of interesting questions.

A. Redox regulation of Rac1

1. Relevance

Rac1 is a small GTPase with its activity regulated through the prototypical nucleotide switch mechanism (*2*). GTP-bound Rac1 populates the activated conformation, and hydrolysis of the γ phosphate inactivates the proteins. Rac1-GDP is in an inactive conformation; as Rac1 binds GTP and GDP with similar affinity, and the in vivo GTP:GDP ratio is typically at least 10:1, GDP must be released to promote the activated form in cells (*1, 19, 20, 27*). The dominant paradigm in the field is that the nucleotide switch is controlled thought GEFs, GAPs, and GDIs (*1, 19, 20, 27, 31*). However, post-translation modifications have also been shown to control Rac1 activity in

cells, such as phosphorylation, ubiquitination, SUMO-lation, and lipid modification (*32, 33, 36, 38*). Moreover, the Campbell lab has demonstrated that radicals and H_2O_2 can enhance the rate of GDP dissociation in Rac1 through Cys18, which would likely promote the activated form of the protein in cells (*13*). This result is unsurprising because Cys18 is in the Rac1 nucleotide binding pocket and forms multiple interactions with the bound nucleotide (*228, 241*). Interestingly, Rac1 is unique in its many interactions with ROS- and RNS-modulating proteins (e.g., Nox, NOS, and SOD) (*4, 6, 208-213*). Further, it directly activates Nox and regulates the activity of NOS (*4, 208-213*). Thus, I was interested in investigating the effect of oxidative modification at this site on Rac1 activation.

2. Summary

While I was developing a method for high levels of site-selectively oxidized Rac1 using glutathione, I noted that I could site-selected oxidize Cys18 when I dropped the pH of the reaction. This observation suggested that Rac1 Cys18 likely has a lowered pKa. Lowered thiol pKas are typical for the players in cell redox homeostasis and signaling. In fact, recent studies have demonstrated that such thiols typically form hydrogen bonds, and these hydrogen bonds are most likely responsible for the lowered the lowered pKa (*203*). It is relevant then that crystal structures of Rac1 depict hydrogen bonds formation between Cys18 and the bound nucleotide (*228, 241*). As I would predict from the glutathiolation studies and crystal structure, when I applied a thiol reactivity experiment to Rac1, I generated a pH titration curve that indicated Rac1 Cys18 has a pKa at 6.8. The typical pKa for a free Cys thiol is 8.6, above the bounds of physiological relevance. The experimentally derived Rac1 Cys18 pKa is well below this value and is within a

physiological pH range; thus, the level of Rac1 Cys18 reactivity may change in response to changing pH conditions in the cell, which are expected in the microenvironments Rac1 is known to localize to (e.g., macrophages).

As the primary goal of these experiments was to generate an oxidative modification at Rac1 Cys18 and test its effect on Rac1 activation, I examined the rate of GDP dissociation for wtRac1, a redox-insensitive variant (Rac1 C18S), a sulfonic acid mimetic (Rac1 C18D), and Rac1 oxidized with glutathione at Cys18 (Rac1S-SG) (*242*). I showed that modification with a residue similar in size and polarity to Rac1 has little effect on the rate of GDP dissociation and, thus, likely the activation status of Rac1 in cells. However, modification to an aspartate, which is a mimetic for a sulfonic acid modification, or glutathiolation at this site produced a dramatically enhanced GDP dissociation rate. These observations suggest that oxidative modification of Rac1 in cells will likely yield the activated form. The Cox lab verified that this hypothesis was correct where they observed enhanced Rac1 activation when they expressed Rac1 C18D compared with wtRac1 and Rac1 C18S.

I also performed $^{1}H/^{15}N$ HSQC experiments for Rac1 C18D and Rac1S-SG, which detect changes in the electrochemical environment of the protein backbone amides. These experiments identified certain residues that are sensitive to oxidation at Cys18. Because Rac1 Cys18 is in the nucleotide binding pocket and directly interacts with the bound nucleotide, I would predict that Cys18 oxidation would perturb residues the nucleotide-sensitive regions of the protein (switch I and II), which was verified through the NMR experiments. However, it is interesting that residues in the insert region were also perturbed upon Cys18 oxidation. It has been shown that the insert region is involved

in effector recognition for certain Rac1/effector interactions, including p67 (the Nox subunit that binds Rac1 for activation) (*205, 206*). Perhaps oxidation differentially affects the interaction between Rac1 and certain effectors. It would be interesting to test Rac1 binding with a series of known effectors to investigate this question.

3. Do additional oxidative modifications alter Rac1 activation?

I have demonstrated that modification of Rac1 Cys18 can impact the level of GDP dissociation. However, not all modifications to this site have the same impact; the Rac1 C18S variant only enhanced GDP dissociation 1.1-fold, but Rac1 C18D increased the rate by ~200-fold. As Rac1 is involved in a host of redox-modulating events in cells and localizes to enzymes that produce various types of ROS and RNS, it is likely that Rac1 can incur multiple types of oxidative modifications. I speculate that the level of Rac1 activation localized to such events is determined by the particular modification that ensues. For example, Rac1 glutathiolated at Cys18 is a form of the protein with a large, negatively charged tripeptide localized to the nucleotide binding pocket, which is important for Rac1 activation. Modification with NO, for example, would produce a form of the protein with a relatively smaller increase in mass at this site compared with glutathiolation. Moreover, an NO modification would not produce a negative charge in the binding pocket but rather a polar environment similar to wtRac1 or Rac1 C18S. Given the earlier observations in the Campbell lab that H_2O_2 produces a 10-fold increase in GDP dissociation (*13*), I would expect that smaller, polar oxidative modifications (e.g., -NO, -OH, and -O_2) would generate a similarly significant, but less dramatic increase in GDP dissociation.

Moreover, the method for site-selective oxidative modification at Rac1 Cys18 that exploits its lowered pKa could also be used to produce different oxidative modifications at Rac1 Cys18, which could then be tested for an altered GDP dissociation rate and used in NMR experiments to discern the regions affected by the various oxidations. Further, the similar GDP dissociation rate increase and residue perturbation for Rac1 C18D and Rac1-GS suggests that it is a useful mimetic for larger, negatively charged modifications in cell-based experiments. A similar parallel may be drawn from biochemical and biophysical characterization of Rac1 C18N and Rac1-NO or Rac1-O_2. I would speculate that such characterization will demonstrate that Rac1 C18N is a good mimetic for the smaller, polar oxidative modifications at Cys18.

4. Do oxidative modifications at Rac1 Cys18 perturb effector binding?

An additional, intriguing observation from the studies described herein is that in addition to the nucleotide-sensitive regions of the protein, which we would predict oxidation at Cys18 to affect, residues in the insert region of Rac1 were also perturbed. Studies have shown that the insert region plays a role in Rac1/effector interactions; I speculate that Rac1 Cys18 oxidation may exert distinctive effects on the interactions between Rac1 and its various effectors. The cell-based studies herein demonstrated that the interaction between Rac1 and effectors with RBD domains may not be affected by oxidation because Rac1 activation in cells was determined by an RBD pull-down experiment. For the Nox/Rac1 interaction, certain studies have observed a dependence on the Rac1 insert domain; however, this finding has been contracted in an additional investigation (*205, 206*). Perhaps the oxidation state of Rac1 plays a role in Nox binding;

as the studies did not examine the oxidation state of Rac1, it is possible that perturbation of this status may underlie the contradictory results.

5. Conclusions

I have demonstrated herein that Rac1 is selectively oxidized at Cys18 because it has a lowered pKa. Further, I have shown that oxidation of Rac1 enhances GDP dissociation ~200-fold. As expected, expression of a sulfonic acid mimetic or the presence of oxidants with wtRac1 produces the activated from of the protein in cells. For this study, I utilized glutathione modification and a sulfonic acid mimetic at Cys18, which are bulkier, negatively charged groups. However, the Rac1 C18S redox-insensitive variant did not have a significantly enhanced GDP dissociation rate, which is expected because the Ser side chain is similar to a thiol in size and polarity. Thus, it is likely that various types of oxidative modification at this site will produce differential results. For example, an -NO or -O_2 modification at this site would introduce a relatively smaller group compared with a glutathione or sulfonic acid modification. Perhaps more importantly, compared with –GS and –O_3, an –NO or –O_2 modification is polar, not negatively charged, which may have a dramatic impact on the level of perturbed nucleotide binding. As Rac1 is localized to enzymes that produce various types of oxidants, it is relevant to investigate the impact of the different types of oxidative modifications Rac1 Cys18 may incur. The method developed for site-selective glutathiolation of Rac1 may be a useful tool for investigating various types of oxidation at this site. Further, as biochemical characterization of the Rac1 C18D variant demonstrated that it was a good mimetic for a bulky, negatively charged oxidation at Cys18, a nice series of experiments may include characterization of Rac1 C18N as a

mimetic for a bulkier, polar oxidative modification at this site in vitro and in cells. In addition, the NMR-based data demonstrate that residues in the switch I and II regions are affected by oxidation, which is expected because these regions are sensitive to the bound nucleotide. Interestingly, the insert region is also affected by oxidation. Certain studies indicate that this region is important for effector recognition. Perhaps only particular Rac1 effector interactions are perturbed by oxidation. Moreover, Rac1/effector interactions may be sensitive to only specific types of oxidative modification. An intriguing future direction would include a series of studies devoted to discerning the impact of various oxidative modifications at this site with different Rac1 effectors.

B. Is SOD a novel Rac1 Effector?

1. Relevance

Rac1 is a GTPase in the Rho subclass of the Ras superfamily. It is a regulator of multiple critical cell processes, and its mutation or dysregulation can generate multiple pathophysiological conditions, such as cancer, cardiovascular disease, and neurological disorders. One of the central functions for Rac1 is regulation of redox homeostasis and signaling. Rac1 directly interacts with major redox-modulating enzymes, including Nox and NOS. Rac1 directly stimulates activation of Nox (which produces superoxide); further, it regulates NOS activity, and at least one report has indicated that Rac1 interaction with NOS can directly promote NOS activity (which generates nitric oxide). A recent study demonstrated that Rac1 interacts with SOD1 in a redox- and nucleotide-dependent manner. SOD1 is a key antioxidant enzyme in the cell, which dismutates the highly reactive superoxide to the less reactive hydrogen peroxide. Moreover, the authors connected the Rac1/SOD1 interaction to ALS through SOD1 variants that are known to

be involved in ALS. Interestingly, Rac1 has been indicated in ALS formation and progression through its interaction with the effector alsin, the mutation of which has been associated with fALS. Given these observations and that the Harraz, et al. study was predominantly cell-based, I was interested in investigating the SOD1/Rac1 interaction using biochemical and biophysical techniques.

2. Summary

Because Rac1 is so heavily involved in fundamental cell function, including redox homeostasis and signaling, I was interested in examining the recent cell-based report that SOD1 and Rac1 interact in a redox- and nucleotide-dependent manner. I used a FRET-based assay to explore whether SOD1 distinctly bound Rac1-GDP and Rac1-GMPPCP. The resulting data produced different binding curves for GDP- and GMPPCP-bound Rac1. Moreover, given Harraz, et al.'s report that this interaction is redox-dependent and the above observations that Rac1 Cys18 affects Rac1 activation as well as perturbs key residues in and around the regions involved in effector interactions, I also tested the effect of Rac1 Cys18 oxidation on SOD1 binding. I noted clear distinctions in the binding curves for Rac1 and SOD1 with varying oxidation states at Cys18. However, these binding assays that demonstrate disparate binding for Rac1 and SOD1 depending on the bound nucleotide and Cys18 oxidation state produced binding curves that indicate greater than one species of Rac1 or SOD1 are present. I further used $^{1}H/^{15}N$ HSQC NMR experiments to study the interaction between Rac1-GDP and SOD1. The data indicate that residues in and around the effector-binding regions of Rac1 are perturbed. Interestingly, the data are primarily line-broadening events, which may indicate low

affinity binding (expected for Rac1-GDP and its effectors) or that the Cu molecule in the SOD1 active site is generating paramagnetic relaxation at certain Rac1 residues.

3. Heterogeneity in nucleotide-dependent binding

One of the key observations was that the binding curves for SOD1 and Rac1 indicate that the interaction may be nucleotide- and Cys18 oxidation-dependent. However, the data comprise an ambiguity, as the binding curves also show two apparent binding events, one low-affinity and one high-affinity. The next step for these experiments should be directed at discerning the source of the ambiguity and validating as well as better quantitating the interaction between Rac1 and SOD1. There are several possibilities for the source of the ambiguity: (i) a heterogeneous Rac1 or SOD1 sample and (ii) an artifact from the assay. Reproduction of the binding curves herein using a separate assay (e.g., isothermal titration calorimetry, ITC) should indicate whether the ambiguity is due to an artifact of the assay. Next, a heterogeneity in the SOD1 sample may also generate the ambiguity apparent in these assays. The SOD1 sample used herein is purified from erythrocytes, which comprise three predominant species; one is unmodified, another is primarily phosphorylated, and a final species is mainly glutathiolated. The SOD1 species are separated through anion exchange and have been previously demonstrated to elute in three different fractions; herein, we use the fraction that elutes first ("peak 1") and was characterized as unmodified in previous studies. However, as the SOD1 fractions are not verified for each sample, the samples that we use may comprise greater than on SOD1 species. Nevertheless, I speculate that inhomogeneity in the Rac1 sample is the mostly likely source of ambiguity. I have shown that Cys18 oxidation is involved in Rac1 activation and the prototypical Rac1 effector-

binding regions are perturbed by the oxidation state of Cys18. Moreover, the various Rac1 Cys18 oxidation samples with SOD1 produced unique binding curves, which suggests that oxidation at this residue plays a role in the SOD1/Rac1 interaction. Most likely, the reducing agents used did not generate an entirely reduced species, which yields two different Rac1 species with distinct binding affinities for SOD1. A series of HSQC experiments using Rac1 C18S and C18D might be useful to discern verify that oxidation at Cys18 alters the interaction between Rac1 and SOD1. Moreover, it might be interesting to test different reducing agents at different concentrations to demonstrate that variations in redox status modulate the Rac1/SOD1 interaction.

4. Redox-dependent binding

In addition to clarifying and verifying the data herein, an additional series of experiments should be directed towards discerning whether specific oxidative modifications at Rac1 Cys18 alter its interaction with SOD1. As discussed above, likely various oxidative modifications at Cys18 will affect Rac1 activation in unique ways. Given that such modifications also perturb the effector-binding regions of Rac1, such modifications may also affect effector interaction in a modification-specific manner. Further, as mentioned above, we have taken advantage of the lowered pKa at Cys18 to produce a Rac1 sample with a significant fraction selectively glutathiolated at Cys18. It would be interesting to perform the binding assays described herein using this species (Rac1S-SG). Moreover, I have proposed that a Cys to Asn variant may be a reasonable mimetic for bulkier and polar oxidative modifications at Cys18 (e.g., -NO and -O2). This mimetic could also be used to examine differences in binding between Rac1 and SOD1 based on various oxidative modifications at Cys18.

5. Regions affected by SOD1 binding

An interesting observation from Harraz, et al. was that the SOD1/Rac1 interaction played a role in Nox-mediated superoxide generation; and this group proposed that SOD1-bound Rac1 promoted Rac1/Nox binding and Nox activation. As Rac1 is known to directly interact with the Nox subunit p67 to facilitate superoxide production, and this interaction has been structurally characterized through crystallography studies, a fascinating avenue of future studies for this interaction may involve structural studies for Rac1 and SOD1. Studies on the Rac1/p67 interaction indicate that residues in the switch I and II regions are involved in this interaction, and residues in the Rac1 insert region may also play a role. Interestingly, these regions were perturbed upon SOD1 binding as determined from the line-broadening observed in the NMR experiments with SOD1 and ^{15}N-labeled Rac1. The following additional structural studies on this interaction may clarify the SOD1 and Rac1 residues involved in this interaction. The line-broadening observed may be generated by low-affinity binding between Rac1-GDP and SOD1, which would be expected in an effector-like interaction. It may also result from paramagnetic relaxation, as SOD1 has a Cu molecule in its binding site. If the line-broadening is due to paramagnetic relaxation, then distance information can be generated for the SOD1 active site and proximal Rac1 residues. Moreover, as an SOD1 Zn/Zn molecule has been generated and characterized, HSQC experiments using this SOD1 variant would be a good control for the paramagnetic relaxation from the Cu molecule, and it may also provide information on the Rac1 residues perturbed from interaction with SOD1.

6. Conclusions

Rac1 is a GTPase involved in many critical cell functions, including redox homeostasis and signaling. It has been shown to directly interact with and control activation of major redox-modulating enzymes in the cell (e.g., Nox and NOS). Recently, Harraz, et al. described a series of cell-based observations that showed Rac1 interacts with SOD1 in a nucleotide- and redox-dependent manner. As I have demonstrated that oxidative modification of Rac1 Cys18 can regulated its activation and perturbs residues involved in effector-binding interactions, I sought to use biochemical and biophysical methods to examine the nucleotide- and Cys18 oxidation-dependence of this interaction. I show that this interaction may be nucleotide-dependent and is altered when Rac1 Cys18 is mutated to a Ser (redox-insensitive) or an Asp (sulfonic acid mimetic); however, the data comprise certain ambiguities that future experiments should be directly towards clarifying. Moreover, structural studies demonstrate that residues commonly involved in Rac1/effector interactions were perturbed upon SOD1 binding. Interestingly, similar regions are involved in the Rac1/p67 interaction, which Harraz, et al. proposed was modulated by Rac1/SOD1 binding. Moreover, I showed that oxidative modification at Cys18 also perturbs residues in this region and propose that distinct types of oxidative modification at Cys18 may produce disparate results in Rac1/effector interactions. It would be intriguing to use additional structural studied to better clarify the nucleotide- and Cys18 oxidation-dependence of the SOD1 and Rac1 interaction (e.g., using paramagnetic relaxation experiments with Cu/Zn SOD1 and HSQC experiments with Zn/Zn SOD1).

C. Conclusions

Rac1 is a ubiquitously expressed small GTPase that is involved in multiple fundamental cell processes, and it plays an unusually strong role in regulating redox homeostasis and signaling (*2*). As an emerging paradigm for proteins involved in redox regulation is the presence of a redox-sensitive thiol with a lowered pKa, I was interested in investigating such characteristics in Rac1 (*203*). The Campbell lab previously demonstrated that Rac1 was sensitive to radicals and H_2O_2 through a Cys residue in its nucleotide binding pocket (*13*). I first showed that Rac1 Cys18 has a lowered pKa, which is consistent with this emerging paradigm. Moreover, I exploited this characteristic to site-selectively modify Rac1 Cys18 with glutathione. Using wtRac1, a redox-insensitive control (Rac1 C18S), a sulfonic acid mimetic (Rac1 C18D), and Rac1S-SG, I further demonstrated that oxidation at this site dramatically enhances GDP dissociation. As expected, in cells, wtRac1 in the presence of oxidants or expression of Rac1 C18D produces the activated form of the protein and an activated phenotype (membrane ruffling). Future investigations should include additional types of oxidative modifications at this site, which may have unique consequences based on the particular characteristics of such modifications (e.g., a polar versus a negatively charged modification). As phosphorylation of Rac1 alters its interactions with only a subset of effectors, it would be interesting to examine the effect of oxidation on various Rac1 effectors, especially given that oxidation perturbs certain residues in regions of Rac1 that are involved in effector recognition.

Further, given the cell-based observations by Harraz, et al. that Rac1 and SOD1 interact in a nucleotide- and redox-dependent manner, I sought to examine this

interaction using biochemical and biophysical techniques. I demonstrate that Rac1 and SOD1 produce unique binding curves for Rac1-GDP and Rac1-GMPPCP (a GTP analog) as well as for distinct Rac1 Cys18 oxidative modifications. However, an apparent ambiguity from such experiments must be addressed in future studies, which may include additional methods for producing an SOD1/Rac1 binding curve under similar nucleotide and Cys18 oxidation conditions. Moreover, I showed that prototypical effector-binding regions in Rac1 are affected by SOD1. However, as the predominant perturbation in Rac1 is line-broadening and SOD1 has a Cu molecule in its active site, it would be interesting to use paramagnetic relaxation experiments and a Zn/Zn SOD1 variant to generate additional information on this interaction.

Rac1 is a unique and widely expressed GTPase in the superfamily of small GTPases with a strong role in regulating redox homeostasis and signaling (among other critical cell processes). The field has only partially uncovered the molecules and mechanisms involved in such regulatory events. I demonstrate herein that Rac1 Cys18 is an important residue in redox-based modulation of Rac1 activation. Moreover, I have provided support for the hypothesis that the Rac1/SOD1 interaction may be nucleotide- and Cys18 oxidation-dependent, and this interaction perturbs Rac1 residues that are typically involved in effector interactions. While certain ambiguities in this interaction remain, I have proposed additional experiments that may further clarify and validate this interaction.

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