

**Is Rac1 Glutathionylated in Intact Cells?**

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**Abstract**

Rac1 is a member of the Ras superfamily and Rho subfamily of small GTP-binding proteins or GTPases. It functions as a molecular switch, cycling between an active (GTP-bound) and inactive (GDP-bound) conformation, to regulate diverse physiological processes within mammalian cells. Active Rac1 signals to downstream effectors to control cellular processes such as membrane ruffling. While the downstream signaling pathways that lead to Rac1 mediated events are reasonably well understood, the upstream events that regulate Rac1 are not well characterized.

The goal of this study is to determine if Rac1 is post-translationally glutathionylated. Glutathione (GSH) is a tripeptide consisting of glutamate, cysteine, and glycine with a free thiol. Glutathionylation is the addition of glutathione to a target protein, via a disulfide bond. Recent studies have shown that Angiotensin II induces the glutathionylation of Ras, which affects its activity. These studies are designed to determine if the Ras-related GTPase, Rac1, is glutathionylated.
NIH 3T3 cells were metabolically labeled with \(^{35}\)S-cysteine to determine if Rac1 is glutathionylated in intact cells. Western Blot and fluorograph analysis revealed that diamide, a chemical that forces the formation of disulfide bonds, can increase the amount of glutathionylated FLAG-epitope tagged Rac1. Preliminary studies indicate that under such conditions, Rac1 is modified by \(^{35}\)S-cysteine in a DTT reversible manner. Future studies will focus on utilizing physiological stimuli to promote and assess reversible glutathionylation of Rac1 in intact cells.

**Introduction**

The Ras superfamily of small guanine nucleotide triphosphate (GTP) binding proteins consists of six subfamilies: Rho, Rap, Ral, Ran, Rab, and Arf. Rho, Rap, and Ral are involved in growth regulation and cell morphology, while Ran is involved in nuclear import, and Rab and Arf in vesicular transport. These small GTP-binding proteins function as molecular switches, cycling between an active (GTP-bound) and inactive (guanine nucleotide diphosphate (GDP) -bound) conformation, to regulate diverse physiological processes within mammalian cells (figure 1). Ras GTP-binding proteins are known to regulate cell growth, cytoskeletal reorganization, and the activation of protein kinases. Although these proteins have the intrinsic ability to activate and deactivate themselves, there are several regulatory proteins that act throughout the GTPase cycle. For example, GTPase activating proteins (GAP) increase the intrinsic GTP hydrolysis rate of these proteins to turn them off. GDP dissociation Inhibitors (GDI) lock the protein in the off position, while guanine nucleotide exchange factors (GEF) promote the exchange of GDP for GTP to activate the protein (Alberts, 2002).
Small GTP-Binding Proteins Function as Molecular Switches

Figure 1: Small GTP-binding proteins function as molecular switches, cycling between the active (GTP-bound) and inactive (GDP-bound) conformation, to regulate diverse physiological processes within mammalian cells. There are several regulatory proteins that facilitate this cycle, such as GAP, GEF, and GDI.
Rac, Rho, and Cdc42 are small Rho GTPases, which are regulators of actin dynamics in motile cells during cell morphogenesis (Hall and Nobes, 2000). Previous studies have indicated Rac, Rho, and Cdc42 are all involved in the formation of actin-based structures. Specifically, Rac is involved in membrane ruffling, the formation of lamellipodia (Nobes and Hall, 1995), and cell motility (Chen et al., 2000). Active Rac1 signals to downstream effectors to control cellular processes such as membrane ruffling. It can be found in the cytosol where it is irreversibly prenylated by geranylgeranyl transferase I (GGTASE I); the addition of this 20 carbon chain renders Rac1 hydrophobic. Prenylated Rac1 can be found in the cytosol and in the plasma membrane, and the prenyl group stabilizes the interaction of the GTPase with the membrane. In the cytosol, Rac1 is found in complex with RhoGDI. This Rho GDP dissociation inhibitor locks the protein in the inactive GDP bound conformation, and serves to mask the hydrophobic prenyl group of Rac1 in the hydrophilic cytosol (Zang, 2000).

While the downstream signaling pathways that lead to Rac1 mediated events are well understood, the regulation of Rac1 is not fully characterized. It is highly likely that there are other post-translational modifications regulate the activity of Rac1. There are many modifications that could have been examined, but this study focused on the possibility of Rac1 being reversibly modified by glutathione (GSH).

Glutathionylation is the addition of glutathione to a protein through a disulfide bond. Glutathione, whose synthesis is catalyzed by ATP hydrolysis, is a tripeptide of glutamate, cysteine, and glycine with a free thiol group. It is the
most abundant low molecular thiol in mammalian cells. In figure 2, you can see that the thiol groups on the cysteine residues of glutathione and another glutathione molecule (or the protein to be modified) each lose a hydrogen atom to form a disulfide bond. Unlike prenylation, glutathionylation is reversible, and glutaredoxin catalyzes the deglutathionylation of proteins (Wang et al., 2003). Glutaredoxin is an electron donor that reduces disulfide bonds, and it is known to have a high level of sequence conservation. Because GSH modification is reversible, it makes it a possible mechanism for protein regulation.

**Glutathione Disulfide Formation**

![Glutathione Disulfide Formation](image)

**Figure 2**: Glutathione is a tripeptide of glutamate, cysteine and glycine with a free thiol group whose synthesis is catalyzed by ATP hydrolysis. Two thiol groups on the cysteine residues are oxidized to form a disulfide bridge (King, 2005).
Glutathionylation appears to play a major role in the regulation of protein function and signal transduction (Cotgreave, 1998). Angiotensin II has been shown to increase glutathionylation of Cys118 and increase the activity of Ras (Adachi et al., 2003). This cysteine residue is in the crucial GTP binding region of the protein. Although Rac1 does not have a cysteine residue in the GTP binding domain, there is a cysteine within ten residues of it, which could affect the conformation of the GTP binding region and, in turn the activity of Rac1 (Adachi et al., 2004). Since Ras is regulated by glutationylation, then it is possible that the related protein, Rac1, is also regulated by GSH modification.

Even more interesting is that when glutaredoxin expression is knocked down in mammalian cells, actin polymerization is inhibited. In a study conducted by Jun Wang et al., a 290 base double stranded RNA was used to knock-out glutaredoxin. Glutaredoxin is an enzyme that has been shown to facilitate the deglutathionylation of proteins. This dsRNA knocks out protein expression by first being cleaved into approximately 22 nucleotide fragments by Dicer, which then binds to RNA-inducing silencing complex (RISC) to unwind the dsRNA. The anti-sense RNA then binds to the mRNA transcript of glutaredoxin, which is then cleaved, preventing translation. The dsRNA was intracellularly generated to allow for better knock down of glutaredoxin. This is achieved by constructing a bidirectional overexpression vector with sense and antisense DNA and strong promoters, and when introduced into the cell, dsRNA will be expressed. They showed that actin deglutathionylation plays a role in actin polymerization, translocation, and reorganization near the cell periphery (Jun Wang et al., 2003).
Their results also support previous indications that glutaredoxin catalyzes the deglutathionylation of protein, such as actin.

Glutathione can protect the cell by scavenging for reactive oxygen species. It provides the reducing equivalents needed for glutathione peroxidase catalyzed reduction of reactive oxygen species such as hydrogen peroxide (figure 3). When mammalian cells are exposed to increased oxidative stress, the ratio of reduced to oxidized GSH (GSH/GSSG) will decrease due to GSSG accumulation. Reactive oxygen species are often generated in response to growth factor stimulation, which in turn, leads to increased levels of tyrosine phosphorylated and glutathionylated proteins.

**Glutathione as an Antioxidant**

\[ \text{H}_2\text{O}_2 + 2\text{GSH} \xrightarrow{\text{GPx}} \text{GSSG} + \text{H}_2\text{O} \]

Figure 3: Glutathione provides the reducing equivalents needed for the glutathione peroxidase (GPx) catalyzed reduction of hydrogen peroxide. This helps to protect the cell from potentially damaging reactive oxygen species.

A study conducted by Jun Wang et. al. investigating the affects of epidermal growth factor (EGF) induced glutathionylation yielded interesting results. They have shown that EGF induces the deglutathionylation of actin, which leads to actin cytoskeletal reorganization. Actin transfers its glutathione to glutaredoxin, which causes the actin to polymerize, but this is surprising because one would think that EGF would induce the opposite affect, increased
glutathionylation. They found that protein-tyrosine phosphatases (PTPs), which catalyze the dephosphorylation of proteins, are glutathionylated under EGF treatment. When protein-tyrosine phosphatases are glutathionylated, they can no longer dephosphorylate proteins. Reactive oxygen species are also known to inhibit their ability to dephosphorylate proteins by converting the active site cysteine into sulfenic acids; these sulfenic acids are easily glutathionylated. The conclusion of this study suggests that the observed deglutathionylation of actin is due to an active compensatory process. The glutathione concentration may not be great enough in the cell to glutationylate both actin and protein-tyrosine phosphatase, so actin is deglutathionylated, leading to actin polymerization (Jun Wang et al., 2001). This is an interesting study because Rac1 is known to be a critical player in actin cytoskeletal polymerization and cell morphology, so it may also be regulated by glutathione.

Many glutathionylated proteins have been identified, but only Ras, actin, and PTP1B are known to be regulated by this modification (Wang et al., 2001). This study focuses on the possible glutathionylation of Rac1 for several reasons. Rac1 is in the same superfamily of Ras, and it is also involved in actin polymerization (active Rac1 signals to downstream effectors to control cellular processes such as membrane ruffling). For these reasons, it is a likely post-translational modification of Rac1. Showing that Rac1 is able to be glutathionylated is the first step in determining if the function of Rac1 is regulated by glutathionylation. The objective of this report was to show that Rac1 is able to
be glutathionylated with the use of diamide, which forces the formation of disulfide bonds.

**Materials and Methods**

**Transfection of NIH 3T3 cells**

NIH 3T3 cells were plated at a density of 600,000 cells/100 mm dish in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were transfected 24 hrs after plating with Lipofectamine Plus using 7.5 µg FLAG Rac1 WT mixed with 12 µl Plus reagent and 18 µl Lipofectamine as directed by Invitrogen. The following day, media was aspirated from cells, 5 ml of DMEM/10% dialyzed FBS/50 µg/ml Cycloheximide/0.1mM Dimethyl fumarate (DMF)/0.1 mM L-buthionine sulfoximine (L-BSO) was added, and the cells were incubated for 90 minutes at 37ºC/5% CO₂. Cells were rinsed twice with 37ºC Dulbecco’s phosphate buffered saline (DPBS).

**Metabolic [³⁵]S-Cysteine Labeling**

To begin labeling, 3 ml of DMEM/10% dialyzed FBS/50 µg/ml Cycloheximide and 90 µCi of [³⁵]S-Cysteine were added to the cells and incubated for 4 hrs at 37ºC/5% CO₂. The radioactivity was aspirated off and cells were rinsed twice with DPBS. Cells were then treated with 3 ml of DMEM/5mM Diamide or 3 ml of DMEM and incubated at 37ºC/5% CO₂ for 10 min. Cells were rinsed and incubated for 5 min. at room temperature with 5 ml of DPBS/50 mM n-ethyl maleimide (NEM). Cells were harvested and pelleted at 1,000rpm/10 min./4ºC. The cell pellet was resuspended in 100 µl RIPA buffer (100 mM Tris pH 8.3; 2 mM EDTA/0.1% SDS; 0.5% DOC; 0.5% NP-40) and diluted with 400 µl
homogenization buffer (50 mM Tris/HCl (pH 7.4)/1 mM EDTA/1 mM EGTA/0.2 mM PMSF/0.1 mM leupeptin). 10% of the diluted sample was removed for Western blot analysis to confirm expression of FLAGRac1. To precipitate FLAGRac1, 150 µl of a 1:4 slurry of anti-FLAG resin (prepared as directed by Sigma) was added to the remaining 90% and rotated at 4ºC for 1 hr. The resin was collected by centrifugation in an Eppendorf microfuge at 10,000rpm/4ºC for 30 s. The supernatant was removed and the resin washed three times with 1.0 ml of ice-cold phosphate buffer (10 mM NaPO$_4$/1 mM MgCl$_2$/200 µM GDP/0.15 M NaCl). FLAGRac1-GSH was eluted by incubating for 5 min. at room temperature with 100 µl of 0.1 M glycine (pH 3.0) and spinning at 4ºC/10,000n for 30 s. To normalize the pH, the eluate was added to a new tube with 10 µl 1 M Tris (pH 8.0) and spun again at 4ºC/10,000n for 30 s. The top 100 µl of eluate was transferred to a new tube. The eluate was then divided into two samples and half were treated with Dithiothreitol (DTT).

**Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS/PAGE)**

Samples were then mixed with SDS sample buffer (6.26 ml 0.5 M Tris 6.8, pH 6.8/5.0 ml 100% glycerol/24 g urea/10 ml 10% SDS/2.5 ml B-mercaptoethanol/diluted to 50 ml with millipore water) and subjected to SDS/PAGE gel. The gel consisted of a 12.5% running gel (6.4 ml millipore water/2.5 ml 0.5 Tris, pH 6.8/1.0 ml 40% acrylamide, Bis/100 µl 10% SDS/100 µl 10% APS/10 µl TEMED) and a 4% stacking gel (17 ml millipore water/10 ml 1.5 Tris, pH 8.8/12.5 ml 40% acrylamide, Bis/400 µl 10% SDS/200 µl 10% APS/20 µl TEMED). The gel was run in a buffer consisting of 120 g Tris base, 576 g
glycine, 40 g SDS diluted to 4 L, and run at a constant 35 mA/100V through the stacker and then increased to a constant 50 mA/210V for 2 hrs through the running gel.

**Flourography**

Proteins were electrophoretically transferred to polyvinylidene fluoride dyneon fluoropolymer (PVDF) transfer membrane in a buffer of sodium bicarbonate 100mM NaHCO₃/30 mM Na₂CO₃ over night at 4ºC. The membrane was incubated with Amplify (Amersham) for 30 min., and subjected to flourography to visualize $^{35}$S labeled cysteine. The membrane was placed into a Biomax LE Screen with Biomax MS film inside a standard cassette. The film was placed at -20ºC for 20 hrs. $^{35}$S signals were quantified with a GS-710 densitometer and Quantity One software (BioRad).

**Western Blot**

The membrane was rewetted with methanol, rinsed with millipore water and incubated at room temperature for 10 min. The membrane was then rinsed again and incubated for an additional 10 min with millipore water. Total protein was then analyzed by Western blotting with anti-FLAG antibody and goat anti-mouse-HRP.

A solution of 0.1% Tween 20 in phosphate buffered saline (PBS-T) and a solution of 0.1% Tween 20 in PBS with 5% dry milk (PBS-TM) were made. The membrane was blocked with 10 ml of PBS-TM for one hr. at room temperature while rocking. The PBS-TM was aspirated off, and the membrane incubated at room temperature with fresh PBS-TM and anti-FLAG antibody (1:10,000 dilution)
for one hr. while rocking. The membrane was washed once with 10 ml of PBS-TM for 15 min. (rocking/room temperature), and twice for 5 min. The secondary antibody, Goat anti-mouse horseradish peroxidase (GAM-HRP) (1:3000 dilution), and 6 ml of PBS-TM was incubated with the membrane for 20 min. (room temperature/rocking). The membrane was again washed with PBS-TM once for 15 min. and twice for 5 min., and then twice again with PBS-T for 5 min. Excess solution was drained off the membrane and briefly (1-2 min.) dried on blotting paper. The membrane was then incubated with 3.5 ml of detection reagent #1 and 3.5 ml of detection reagent #2 (Amersham Pharmacia) for one min. undisturbed. The membrane was dried as previously and wrapped in saran wrap. The blot was then placed in a cassette and film placed down on the blot in a dark room. The film was exposed for one min. Protein was then quantified from the developed film with a GS-710 densitometer and Quantity One software (BioRad).

**Results and Discussion**

Flourography is a method that is used to visualize radioactively labeled substances in gels, blots, or other biochemical separations. A radioisotope is an atom that decays over time due to its unstable nuclei, and these are often used to track molecules in biological systems. In this experiments, flourography was used to visualize the $^{[35]}$S labeled cysteine. The radiolabeled cysteine emits radiation, which excites a fluor, and when this fluor relaxes back to its ground state, a photon of light is emitted. This emission of light is detected by the film, so when the film is developed, one can visualize where on the membrane the
$^{[35]}$S labeled cysteine is located (Waterborg et al., 1994). Autoradiography is a technique where the film is put down without treating the membrane first. Fluoroagraphy is different from autoradiography because the membrane is first amplified and then put into a screen, which reflects the signal repeatedly through the film. This technique is useful for weaker signals, because fluorography can increase the sensitivity to weak radiation signals by up to ten fold for the $^{[35]}$S isotope (Laskey, 2003).

Although treatment with cycloheximide is intended to shut down $^{[35]}$S cysteine backbone incorporation, it is not completely successful. The fluoroagraphy will identify not only glutathionylated FLAGRac1, but other proteins that have incorporated $^{[35]}$S cysteine residues into their backbones as well. After the radiolabeled cysteine is identified, a Western blot is utilized to determine where on the membrane the FLAGRac1 of interest is located. The signal from $^{[35]}$S cysteine must line up with the protein band identified as FLAGRac1, because some contaminates are present even after precipitating the protein.

The FLAGRac1 can be identified from the Western Blot. This is possible because the primary antibody, anti-FLAG, binds to the FLAG tag of Rac1 on the blot. The secondary antibody, GAM-HRP, then binds to the primary antibody. The detection reagents can then locate where this secondary antibody is located on the blot. This technique employs enhanced chemiluminescence (ECL). The first detection reagent binds to the secondary antibody; when the second detection reagent is added, it reacts with the first, and gives off light. In this
experiment, when the Lumigen PS-3 Acridan substrate is oxidized, it gives off acridinium ester intermediates. Under slightly alkaline conditions, these intermediates react with peroxidase, which produces a sustained, high intensity chemiluminescence at 430 nm (figure 4; Amersham Biosciences, 2002).

Diamide forces the glutathionylation of available cysteine residues, and our data suggests that diamide did induce glutathionylation of Rac1. Diamide is a small thiol oxidant that reacts readily with glutathione to force the formation of disulfide bonds. It is a diazene dicarbonyl compound, and the reaction of diamide with thiols can be broken into two steps. First, a sulfenylhydrazine is
formed when a glutathiol radical adds to the double bond of the diazene. The sulfenylhydrazine then reacts with another glutathiol radical producing a hydrazine and a glutathione disulfide (figure 5). This reaction takes place more rapidly with GSH because glutathione is the most abundant non-protein thiol in mammalian cells. Diamide can also force a protein-GSH disulfide bond, although this process happens more slowly than the formation of glutathione disulfide. Diamide more readily reacts with a small thiol like GSH than with a protein due to steric hindrance (Rovinsky, 2001).

**Reaction of Diamide with Glutathione**

![Chemical structure diagram]

Figure 5: The reaction of diamide with glutathione occurs in two steps. A sulfenylhydrazine is formed when a glutathiol radical (GS-) adds to the double bond of diazene. The product then reacts with another GS- anion to produce hydrazine and glutathione disulfide.

Densitometry was used to determine the ratios of total FLAGRac1 from the Western blot, to that of the fluorograph, which identifies the $^{[35]}$S cysteine. Figure 6 shows the percentage of total FLAGRac1 incorporating $^{[35]}$S cysteine. Dithiothreitol (DTT) breaks disulfide bonds, which will release the GSH, but the $^{[35]}$S cysteine incorporated into the backbone of FLAGRac1 will not be released. The remaining signal is associated with backbone incorporation. In the plus
diamide condition, there is a significant decrease in $^{35}$S cysteine with the addition of DTT, which indicates that glutathione was released. In the minus diamide condition, the amount of releasable $^{35}$S cysteine decreases in comparison to the plus diamide condition. Therefore, the data indicates that diamide increases the glutathionylation of Rac1.

**Figure 6:** Percentage of total FLAGRac1 incorporating $^{35}$S-cysteine. Dithiothreitol (DTT) was added to plus and minus diamide conditions, and densitometry was used to determine intensities.

**Future Studies**

This study has shown that diamide increases the glutathionylation of Rac1, but it may not be physiologically relevant. Showing that Rac1 can be glutathionylated by forcing disulfide bond formation with diamide is merely the first step in determining if Rac1 is glutathionylated *in vivo* and regulates its
function. Physiological studies will result in much smaller signals, so this study was an effort to optimize the method.

To study if the glutathionylation of Rac1 is physiologically relevant, physiological stimuli such as growth factors must be used to induce GSH modification of Rac1. The affects that glutathionylated Rac1 has on the cell can then be observed, and specifically if it affects the glutathionylation state of actin. If it is shown that there is a significant affect on the cell when altering the glutathionylation state of Rac1, it can then be assumed that glutathione regulates the function of the protein.

Since physiological studies would result in smaller signals that would be difficult to visualize with physiologically stimulated glutathionylation, increased Rac1 expression would be needed. An adenovirus system is currently being developed to increase the expression of FLAGRac1 in hopes of increasing the $^{[35]}$S cysteine signal. Adenoviruses have several advantages over the Lipofectamine-Plus method used in this study to transfect cells. Adenoviruses can infect most cell types and both replicative and non-replicative mammalian cells. This is important because the virus could be used to study many different cells types. The virus is much more efficient at gene transfer and has higher expression than the Lipofectamine-Plus method. The DNA remains as an episome (does not integrate into the host chromosome), and therefore it is unlikely that it will affect the gene expression of other genes. This is important because changing the expression of other genes would introduce error into the system that would not be easily accounted for (Vector Biolabs, 2005).
References


