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A Molecular Redox Switch on p21^{ras}

STRUCTURAL BASIS FOR THE NITRIC OXIDE-p21^{ras} INTERACTION*

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We have identified the site of molecular interaction between nitric oxide (NO) and p21^{ras} responsible for initiation of signal transduction. We found that p21^{ras} was singly *S*-nitrosylated and localized this modification to a fragment of p21^{ras} containing Cys^{118}. A mutant form of p21^{ras}, in which Cys^{118} was changed to a serine residue and termed p21^{ras}C118S, was not *S*-nitrosylated. NO-related species stimulated guanine nucleotide exchange on wild-type p21^{ras}, resulting in an active form, but not on p21^{ras}C118S. Furthermore, in contrast to parental Jurkat T cells, NO-related species did not stimulate mitogen-activated protein kinase activity in cells transfected with p21^{ras}C118S. These data indicate that Cys^{118} is a critical site of redox regulation of p21^{ras}, and *S*-nitrosylation of this residue triggers guanine nucleotide exchange and downstream signaling.

It is well known that signal transduction pathways initiated by extracellular ligands are dependent on protein-protein interactions for propagation and amplification of their signal. Many of these interactions lead to phosphorylation events. For example, receptor-tyrosine kinases require a series of protein interactions utilizing SH2 and SH3 domains of adaptor proteins to generate an activated form of p21^{ras}, a critical signaling enzyme (1–3).

Recent studies have identified reactive free radicals as central participants in certain signaling events (4–7). Enhancing free radical destruction, either enzymatically or chemically, prevented ligand-stimulated transcription factor (8) and mitogen-activated protein (MAP)[1] kinase (4) activation and also prevented smooth muscle cell mitogenesis and chemotaxis (4). Thus, a role is emerging for reactive free radicals in mediating signal transduction.

Among the many recently discovered functions of NO, a role in signaling has surfaced (9). Although soluble guanylyl cyclase is an important target of NO in mediating some of its physiologic functions such as the regulation of blood pressure (10, 11), other signaling events, some culminating in transcriptional activation, may be cGMP-independent (12–15). Our studies have focused on how NO initiates cGMP-independent signaling within cells (16, 17). We have identified p21^{ras} as a critical target of NO and other redox modulators (17–19). Here, we sought an understanding of the structural basis of the NO-p21^{ras} interaction in the hope of gaining insight into how redox signaling is achieved.

MATERIALS AND METHODS

Preparation of p21^{ras} Proteins—p21^{ras}(1–166) was expressed and purified as described previously (20). p21^{ras}C118S(1–166) was expressed and purified similarly.

Generation of p21^{ras}C118S cDNA Constructs—Codon 118 of truncated (codons 1–166) Ha-*ras* cDNA was mutated from TGT (cysteine) to TCT (serine) using the polymerase chain reaction. The generated cDNA fragment was then sequenced (Sequenase) and cloned into the pATras bacterial expression vector. To generate full-length p21^{ras}C118S an *Nco*I/*Bam*HI fragment (encoding residues 111–166 of the *ras*(1–166) mutant) was exchanged for a 0.8-kilobase fragment encoding residues 111–189 plus 3′-noncoding region and the coding junction sequenced. A *Bgl*II/*Bam*HI fragment encoding full-length Ras(C118S) was then subcloned into the *Bam*HI site of the pCDNA3 mammalian expression plasmid and orientation confirmed by *Bst*XI digestion.

CNBr Digestion and ESI-MS Analysis of p21^{ras} —One small crystal of CNBr (Fluka) was added to 100 pmol of p21^{ras} in 20 μl of 0.1 N HCl in a 0.5-ml polypropylene tube. Digestion was carried out at room temperature for 10 min prior to analysis by ESI-MS. After 10 min, samples were directly electrosprayed into a Finnigan-MAT TSQ-700 triple quadrupole instrument for analysis of *S*-nitrosylation exactly as we described previously (21).

Preparation of NO Solutions—NO solutions were prepared as we described previously (17). Briefly, a solution of 20 mM ammonium bicarbonate solution, pH 8.0, in a rubber-stoppered tube was sparged for 15 min with N_2 and then 15 min with NO gas (Matheson Gas, East Rutherford, NJ). This resulted in a saturated solution of NO (1.25 mM). This solution also contained higher oxides of NO which were not quantified.

GTPase Assay—GDP-preloaded p21^{ras} or p21^{ras}C118S was analyzed for guanine nucleotide exchange activity as we described previously (17). Basal rates of hydrolysis of [γ-^{32}P]GTP were 24.6 ± 6 fmol of PO_4^- released/min/mg for the wild-type enzyme and 18.4 ± 5 fmol/min/mg for p21^{ras}C118S.

Cell Transfection and Culture—The human T cell line Jurkat was grown in RPMI 1640 containing 2% L-glutamine and 10% fetal calf serum. For transfection cells were washed in serum-free medium and resuspended to 1–5 × 10^6 cells/ml in serum-free medium. Liposomes (50 μl of Lipofectin, Life Technologies, Inc.) were mixed with a solution of 10 μg of p21^{ras}C118S plasmid in 150 μl of sterile water. This was then added to 1 ml of culture and placed into culture flasks. After incubation for 24 h at 37 °C and 5% CO_2, the cells were supplemented with 3 ml of selection medium (RPMI 1640 containing 10% fetal calf serum, 2% L-glutamine, and 1 mg/ml G418 (Life Technologies, Inc.)). After another 24 h, cells were resuspended and maintained in the selection medium. Mock transfected cells did not receive any DNA. After 3–4 weeks in selection medium, transfected cells were analyzed for p21^{ras} levels via Western blotting.

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[1] The abbreviations used are: MAP, mitogen-activated protein; NO, nitric oxide; ESI-MS, electrospray ionization-mass spectrometry; ERK, extracellular signal-regulated kinase; PDGF, platelet-derived growth factor.

Fragment	Calc. Mass (Da)
1	7203
2	4540
3	6225

FIG. 1. **Predicted CNBr fragmentation and location of Cys residues of p21^{ras}.** The sequence of p21^{ras}(1–166) is indicated. Cleavage at Met residues with CNBr (indicated by *arrowheads*) yields three major fragments. Fragment 1 (*green underline*) contains Cys^{51} and has a mass of 7,203 Da. Fragment 2 (*purple underline*) contains Cys^{80} and has a mass of 4,540 Da. Fragment 3 (*red underline*) contains Cys^{118} and has a mass of 6,225 Da.

MAP Kinase Activity—MAP kinase activity was measured in an *in vitro* kinase assay using myelin basic protein as a substrate, as we described previously (18). Briefly, serum-starved cells (24 h, 5×10^6) were treated for 30 min at 37 °C, pelleted, and then resuspended in 300 μl of RIPA buffer containing 20 mM Tris, pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 1 mM $NaVO_3$, 2 mM $NaPP_i$, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin. Samples were vortexed, left on ice for 15 min, and then microcentrifuged for 2 min. Protein A-Sepharose prebound to anti-ERK1 or ERK2 (Santa Cruz Biotechnology) was added to supernatants (5 μg/sample). After 1 h at 4 °C, samples were washed twice with RIPA buffer and twice with kinase buffer (25 mM Hepes, pH 7.4, 25 mM β-glcerophosphate, 25 mM $MgCl_2$, 2 mM dithiothreitol, and 0.1 mM $NaVO_3$). After the final wash, samples were resuspended in 20 μl of kinase buffer, and 1 μg of myelin basic protein was added along with 22 μl of 10 μCi/nmol [γ-^{32}P]ATP. After 20 min at 30 °C, 4 μl of 6 × Laemmli sample buffer containing 100 mM dithiothreitol was added, and samples were boiled for 2 min. Samples were run on 15% sodium dodecyl sulfate-polyacrylamide gels and were analyzed via a PhosphorImager (Molecular Dynamics).

RESULTS

Localization of the Site of S-Nitrosylation on p21^{ras}—Our earlier studies correlated a single *S*-nitrosylation event on full-length p21^{ras} with enhanced guanine nucleotide exchange (17). In the present *in vitro* studies, p21^{ras} lacking the carboxyl-terminal 23 amino acids is used. This form of p21^{ras} is commonly used for *in vitro* studies and possesses biochemical activity identical to that of the wild-type enzyme (22). To identify the exact site of *S*-nitrosylation, we took advantage of the fact that cleavage of p21^{ras} at Met residues with CNBr yields three major fragments each containing a single Cys residue (Fig. 1). We monitored each of the Cys residues for *S*-nitrosylation by subjecting p21^{ras} to CNBr digestion followed by analysis using ESI-MS. In this ESI-MS assay, the molecular mass of samples treated with NO is compared with that of untreated samples. An increase in mass of 29 ± 1 Da (the mass of NO, 30 Da, minus the mass of the substituted proton) and its lability to increased energy input are indicative of *S*-nitrosylation (21). As seen in Table I, CNBr digestion of p21^{ras} yielded a fragment with a molecular mass of 6,223 ± 2 Da, corresponding to Fragment 3

TABLE I
ESI-MS analysis of various p21^{ras} preparations

Analyte	Molecular mass	Difference[a]
	Da	*Da*
CNBr-cleaved p21^{ras}	6,223 ± 2	
CNBr-cleaved p21^{ras} + NO	6,253 ± 2	30
p21^{ras}	18,852 ± 2	
p21^{ras} + NO	18,882 ± 2	30
p21^{ras} (C118S)	18,836 ± 2	
p21^{ras} (C118S) NO	18,836 ± 2	0

[a] Difference refers to the mass difference between the untreated and NO-treated preparations within each group.

(Fig. 1). Upon treatment of p21^{ras} with NO and subsequent cleavage with CNBr, Fragment 3 had a new mass clearly indicative of *S*-nitrosylation (Table I); that is, the mass of Fragment 3 from NO-treated p21^{ras} was equal to that of the unmodified Fragment 3 (6,223 Da) plus that of NO (30 Da). This fragment contains Cys^{118}, and thus this Cys residue is the likely target of NO. It is possible that Fragments 1 and 2 (Fig. 1) were not observed because of inter- and intramolecular hydrophobic interactions that precluded their solubilization.

To confirm that Cys^{118} was indeed the site of *S*-nitrosylation, we generated a form of p21^{ras} identical to the wild-type enzyme, except that Cys^{118} was modified to a Ser residue (referred to as p21^{ras}C118S). This modification only changes the sulfur atom of Cys^{118} to oxygen, thus reducing the mass of the enzyme by 16 Da to 18,836 Da. We treated wild-type p21^{ras} with NO under conditions in which we achieved approximately 50% *S*-nitrosylation. Analysis by ESI-MS revealed the parent enzyme (mass = 18,852 Da) and a singly *S*-nitrosylated derivative (mass = 18,882 Da, Table I). Treatment of p21^{ras}C118S with NO under identical conditions resulted in no *S*-nitrosylated product but only the parent enzyme (Table I). These data identify Cys^{118} as the molecular target of NO on p21^{ras}.

Cys^{118} as the Molecular Trigger for NO Signaling—Having established that Cys^{118} is the site of interaction of NO on p21^{ras}, we examined whether this *S*-nitrosylation was responsible for NO-induced p21^{ras} activation and subsequent downstream signaling events. We have found previously that NO and other free radicals induce guanine nucleotide exchange on p21^{ras} in cells and *in vitro* (17–19). Therefore, we examined whether NO could induce nucleotide exchange on GDP-preloaded p21^{ras}C118S *in vitro*. Direct measurement of exchange relies on a filter binding assay to which our p21^{ras}(1–166) protein does not bind quantitatively (22, 23). Therefore, to measure exchange, GDP-preloaded p21^{ras} is treated with NO in the presence of [γ-^{32}P]GTP, and hydrolyzed $^{32}P_i$ is quantified. We have previously shown this to be a measure of exchange in our system (17). As seen in Fig. 2, NO potently stimulated [γ-^{32}P]GTP hydrolysis of GDP-preloaded wild-type p21^{ras} (*open circles*). In contrast, NO had almost no effect on the exchange rate of p21^{ras}C118S (Fig. 2, *closed circles*). The basal rates of hydrolysis for the two enzymes were similar (24.6 ± 6 fmol of PO_4^- released/min/mg for the wild-type enzyme and 18.4 ± 5 fmol/min/mg for p21^{ras}C118S). A clear biphasic curve of activation and subsequent inhibition by higher concentrations of NO was seen. We and others have previously seen this type of biphasic behavior of NO in many systems (15, 17, 24). The inhibitory component may be due to nonspecific noxious effects of high concentrations of NO and its higher oxides or due to quenching (25). These data indicate that interaction of NO with Cys^{118} is required for NO-induced guanine nucleotide exchange on p21^{ras}.

Since NO and other redox modulators can stimulate several biochemical events downstream of p21^{ras}, such as nuclear factor κB translocation and MAP kinase activity (15, 18, 26), we

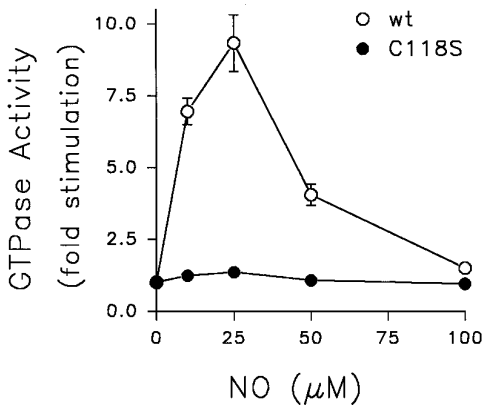

FIG. 2. **Effect of NO on GTPase activity of p21^{ras} and p21^{ras}C118S.** GDP-preloaded wild-type (*wt*) p21^{ras} or p21^{ras}C118S was exposed to the indicated concentrations of NO for 10 min prior to assay of [γ-^{32}]GTP hydrolysis as described under "Materials and Methods." Basal rates for the two enzymes were 24.6 ± 6 fmol of PO_4^- released/min/mg for the wild-type enzyme and 18.4 ± 5 fmol/min/mg for p21^{ras}C118S. Data represent mean ± S.D. of three experiments.

examined whether Cys^{118} on p21^{ras} is a target for NO in cells. Using Jurkat T cells mock transfected or stably transfected with an expression plasmid encoding a full-length version of p21^{ras}C118S (*i.e.* residues 1–189), we examined the ability of NO to activate MAP kinase activity. Immunoprecipitation of the MAP kinases ERK1 and ERK2 from wild-type cells treated with NO-generating compounds *S*-nitroso-*N*-acetylpenicillamine or sodium nitroprusside resulted in enhanced phosphorylation of the ERK substrate (Fig. 3*A*, *open bars*). In contrast, Jurkat T cells stably expressing p21^{ras}C118S(1–189) did not respond to NO in this *in vitro* kinase assay (Fig. 3*A*, *hatched bars*). These transfected cells expressed 7–10-fold more p21^{ras} than the wild-type cells as determined by Western blotting with anti-p21^{ras} antibody, Y13-259 (Fig. 3*B*). This antibody cannot distinguish between wild-type and p21^{ras}C118S, suggesting that although endogenous p21^{ras} was not specifically inhibited, ectopic expression of high levels of mutant p21^{ras} apparently prevented its signaling. This dominant negative activity of p21^{ras}C118S toward NO action may be due to its high level of expression. The pool of effectors available for wild-type p21^{ras} to interact with may be reduced greatly by overexpression of p21^{ras}C118S, perhaps due to their sequestration. Another possibility is that MAP kinase activity is suppressed in the mutant cells. To test this, we treated parental and transfected cells with phorbol myristate acetate (100 ng/ml) and the calcium ionophore A23187 (500 ng/ml) for 5 min. We found that these agents, which bypass p21^{ras} in activating MAP kinase, stimulated MAP kinase activity in both cell types (*i.e.* 221 ± 8 *versus* 261 ± 9% of control, parental *versus* transfected). Thus, NO donors did not stimulate MAP kinase activity in p21^{ras}C118S-transfected cells although these cell harbored a functional MAP kinase system. These data indicate that Cys^{118} is indeed the target of NO on p21^{ras} responsible for triggering downstream signal transduction.

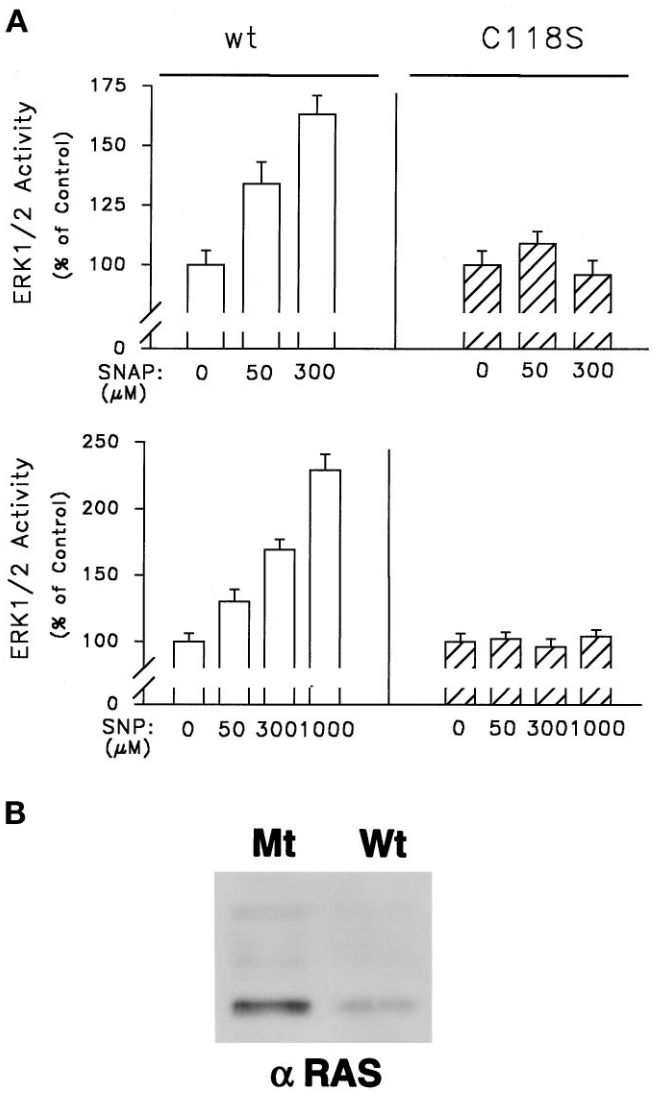

FIG. 3. **Effect of NO on MAP kinase activity in parental or transfected Jurkat cells.** *Panel A*, mock transfected Jurkat cells (*wt*, *open bars*) or Jurkat cells stably transfected with p21^{ras}C118S (*hatched bars*) were treated with either *S*-nitroso-*N*-acetylpenicillamine (SNAP) for 10 min or sodium nitroprusside (SNP) for 30 min prior to lysis and analysis of ERK1 and ERK2 activities as described under "Materials and Methods." Data represent mean ± S.D. of three experiments. *Panel B*, cells (from *panel A*) were lysed, run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western blotted with an anti-*ras* antibody. *Mt*, p21^{ras}C118S-expressing cells; *Wt*, parental wild-type cells.

DISCUSSION

Redox regulation of signaling pathways currently presents several conceptual riddles. These include identifying the source of regulatory redox species, maintaining specificity, and identifying the redox target. Our earlier work demonstrated a functional requirement for p21^{ras} in NO signaling (17). Here, we have focused on identifying the molecular target of NO on p21^{ras}. We have identified Cys^{118} as the critical site of *S*-nitrosylation. The crystal structure of p21^{ras} is well defined (27, 28), and modeling studies show that Cys^{118} is the most surface accessible of the three Cys residues in our p21^{ras} preparation. Using the program X-PLOR (Molecular Simulations, Inc.) the solvent accessible surface of p21^{ras} complexed to GDP (coordinates obtained from the NMR solution structure) was calculated using a 1.4 Å radius probe. Whereas the solvent accessibility of Cys^{51} and Cys^{80} side chains were similar and fairly shielded from the solvent, Cys^{118} was solvent exposed. The buried nature of residues Cys^{80} and Cys^{51} provides a structural basis of why a single *S*-nitrosylation occurs on p21^{ras} upon exposure to NO.

A mechanistic understanding of how *S*-nitrosylated Cys^{118} leads to enhanced guanine nucleotide exchange will likely be provided by solving its x-ray crystal structure. However, some insight is gained from considering what is currently known about p21^{ras} structure and function. Cys^{118} is located within a highly conserved region (NK*X*D) of the *ras* superfamily and indeed all GTP-binding protein sequences. This NK*X*D motif,

in which Cys^{118} is the variable *X* residue in $p21^{ras}$, interacts directly with the guanine nucleotide ring of GTP and GDP and with other nucleotide-binding loops of the protein (29). Independent mutation of residue 116, 117, or 119 leads to an increased dissociation rate of bound nucleotide, resulting in an increased rate of nucleotide exchange (30). Although our $p21^{ras}$C118S mutant had basal rates of guanine nucleotide exchange similar to those of the wild-type protein, it is possible that *S*-nitrosylation results in an alteration in protein-GDP contact, resulting in nucleotide exchange.

The presence of a redox-active residue in such a critical domain suggests that its conservation may reveal enzymes and transductional systems that may be similarly regulated. In the *ras* superfamily, Ha-, Ki-, and N-*ras*, *rap1A*, *rap1B*, *rab1*, and *rab3* contain a Cys residue in this conserved region. In contrast, *ral*, *tc21*, R-*ras*, *rap2*, and *rho* gene products do not. Within the *ras* subfamily, this Cys residue is conserved from slime mold to man. Such conservation suggests that this molecular redox trigger is an important mechanism by which cells respond to reactive free radicals.

It is likely that this molecular switch is regulated by cellular antioxidant levels. We have shown previously that reducing cellular glutathione levels renders the $p21^{ras}$ signaling pathway dramatically more sensitive to redox activation (18). According to this model, as glutathione is oxidized, the Cys switch on $p21^{ras}$ becomes available for modification, and a preprogrammed signaling cascade is initiated. Glutathione or other low molecular weight thiols likely play an important role in *S*-nitrosylation of $p21^{ras}$. NO, under anaerobic conditions, cannot participate in *S*-nitrosylation. NO must be converted to a redox active form such as nitrosonium ion (NO^+; Ref. 9). This occurs rapidly in the presence of metals or thiols, and thus *S*-nitrosoglutathione may be a crucial reservoir of redox active NO.

Such a redox-triggered signaling pathway may also provide a mechanistic understanding of some recent observations identifying a requirement for reactive free radicals in mediating platelet-derived growth factor (PDGF) signaling. In those studies (4), PDGF stimulated H_2O_2 production in vascular smooth muscle cells. When H_2O_2 production was blocked, PDGF-induced enhancement of MAP kinase activity, chemotaxis, and DNA synthesis was prevented. Many of these PDGF-dependent events require $p21^{ras}$, and thus the redox-sensitive target, Cys^{118}, is likely to be involved in this reactive free radical-dependent signaling cascade. Modification of Cys by redox modulators other than NO, such as hydroxyl radical or heavy metals, would necessitate a Cys modification that is chemically different from the *S*-nitrosylation described herein. However, the structural alterations may ultimately be the same.

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