A Molecular Redox Switch on p21ras

STRUCTURAL BASIS FOR THE NITRIC OXIDE-p21ras INTERACTION

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We have identified the site of molecular interaction between nitric oxide (NO) and p21ras responsible for initiation of signal transduction. We found that p21ras was singly S-nitrosylated and localized this modification to a fragment of p21ras containing Cys118. A mutant form of p21ras, in which Cys118 was changed to a serine residue and termed p21rasC118S, was not S-nitrosylated. NO-related species stimulated guanine nucleotide exchange on wild-type p21ras, resulting in an active form, but not on p21rasC118S. Furthermore, in contrast to parental Jurkat T cells, NO-related species did not stimulate mitogen-activated protein kinase activity in cells transfected with p21rasC118S. These data indicate that Cys118 is a critical site of redox regulation of p21ras, 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 for propagation and amplification of their signal. Thus, a role is emerging for reactive free radicals in mediating certain signaling events (4–7). Enhancing free radical destruction, either enzymatically or chemically, prevented ligand-stimulated transcription factor (8) and mitogen-activated protein kinase activity (9) in HL-60 cells. Enhanced free radical destruction, either enzymatically or chemically, prevented ligand-stimulated transcription factor (8) and mitogen-activated protein kinase activity (9) in HL-60 cells. 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 p21ras as a critical target of NO and other redox modulators (17–19). Here, we sought an understanding of the structural basis of the NO-p21ras interaction in the hope of gaining insight into how redox signaling is achieved.

MATERIALS AND METHODS

Preparation of p21ras Proteins—p21ras(1–166) was expressed and purified as described previously (20). p21rasC118S(1–166) was expressed and purified similarly.

Generation of p21rasC118S 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 pOTras bacterial expression vector. To generate full-length p21rasC118S an Ncol/BamHI 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 BglII/BamHI fragment encoding full-length RasC118S was then subcloned into the BamHI site of the pCDNA3 mammalian expression plasmid and orientation confirmed by BamHI digestion.

CNBr Digestion and ESI-MS Analysis of p21ras—One small crystal of CNBr (Fluka) was added to 100 pmol of p21ras(1–166) in 20 μL of 0.1 M 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-nitrosylated exactly as we described previously (21).

Preparation of NO Solutions—NO solutions were prepared as 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 N2 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 p21ras or p21rasC118S was analyzed for guanine nucleotide exchange activity as we described previously (17). Basal rates of hydrolysis of [γ-32P]GTP were 24.6 ± 6 fmol of P2O8 released/min/mg for the wild-type enzyme and 18.4 ± 5 fmol/min/mg for p21rasC118S.

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 1×106 cells/ml in serum-free medium. Liposomes (50 μL of Lipofectin, Life Technologies, Inc.) were mixed with a solution of 10 μg of p21rasC118S 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% CO2, 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 p21ras levels via Western blotting.
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 μM 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 NaVO₃, 2 mM NaF, 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-glycerophosphate, 25 mM MgCl₂, 2 mM dithiothreitol, and 0.1 mM NaVO₃). 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 μM of 10 μCi/mmol [γ-32P]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 Phosphor-Imager (Molecular Dynamics).

RESULTS

Localization of the Site of S-Nitrosylation on p21
—— Our earlier studies correlated a single S-nitrosylation event on full-length p21ras with enhanced guanine nucleotide exchange (17). In the present in vitro studies, p21ras lacking the carboxy-terminal 23 amino acids is used. This form of p21ras 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 p21ras 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 p21ras 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 p21ras yielded a fragment with a molecular mass of 6,223 ± 2 Da, corresponding to Fragment 3 (Fig. 1). Upon treatment of p21ras 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 p21ras was equal to that of the unmodified Fragment 3 (6,223 Da) plus that of NO (30 Da). This fragment contains Cys₁¹⁸, 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 Cys₁¹⁸ was indeed the site of S-nitrosylation, we generated a form of p21ras identical to the wild-type enzyme, except that Cys₁¹⁸ was modified to a Ser residue (referred to as p21ras(C118S)). This modification only changes the sulfur atom of Cys₁¹⁸ to oxygen, thus reducing the mass of the enzyme by 16 Da to 18,836 Da. We treated wild-type p21ras 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 p21ras(C118S) with NO under identical conditions resulted in no S-nitrosylated product but only the parent enzyme (Table I). These data identify Cys₁¹⁸ as the molecular target of NO on p21ras.

Cys₁¹⁸ as the Molecular Trigger for NO Signaling—Having established that Cys₁¹⁸ is the site of interaction of NO on p21ras, we examined whether this S-nitrosylation was responsible for NO-induced p21ras activation and subsequent downstream signaling events. We have found previously that NO and other free radicals induce guanine nucleotide exchange on p21ras in cells and in vitro (17–19). Therefore, we examined whether NO could induce nucleotide exchange on GDP-preloaded p21ras(C118S) in vitro. Direct measurement of exchange relies on a filter binding assay to which our p21ras(1–166) protein does not bind quantitatively (22, 23). Therefore, to measure exchange, GDP-preloaded p21ras is treated with NO in the presence of [γ-32P]GTP, and hydrolyzed 32P, 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-32P]GTP hydrolysis of GDP-preloaded wild-type p21ras (open circles). In contrast, NO had almost no effect on the exchange rate of p21ras(C118S) (Fig. 2, closed circles). The basal rates of hydrolysis for the two enzymes were similar (24.6 ± 6 fmol of PO₄ released/minute for the wild-type enzyme and 18.4 ± 5 fmol/minute for p21ras(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 norelaxation effects of high concentrations of NO and its higher oxides or due to quenching (25). These data indicate that interaction of NO with Cys₁¹⁸ is required for NO-induced guanine nucleotide exchange on p21ras.

Since NO and other redox modulators can stimulate several biochemical events downstream of p21ras, such as nuclear factor κB translocation and MAP kinase activity (15, 18, 26), we
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$^{ras}$ (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. 3A, open 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. 3B). This antibody cannot distinguish between wild-type and p21$^{ras}$, 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$^{ras}$ 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$^{ras}$, 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$^{ras}$-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.

**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$^{99}$ side chains were similar and fairly shielded from the solvent, Cys$^{118}$ was solvent exposed. The buried nature of residues Cys$^{51}$ 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 (NKXD) of the ras superfamily and indeed all GTP-binding protein sequences. This NKXD 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 antioxidants 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.

REFERENCES
1. Bokoch, G. M., and Der, C. J. (1993) *FASEB J.* 7, 750–759
2. Skolnik, E. Y., Li, N., Lee, C.-H., Lowenstein, E., Mohammadi, M., Margolis, B., and Schlessinger, J. (1993) *Science* 260, 1953–1955
3. Satoh, T., Nakafuku, M., and Kaziro, Y. (1992) *J. Biol. Chem.* 267, 24414–24419
4. Sundaresan, M., Yu, Z.-X., Ferrans, V. J., Irani, K., and Finkel, T. (1995) *Science* 270, 296–299
5. Feng, L., Xia, Y., Garcia, G. E., Hwang, D., and Wilson, C. B. (1995) *J. Clin. Invest.* 95, 1669–1675
6. Lo, Y. C., and Cruz, T. F. (1995) *J. Biol. Chem.* 270, 11727–11730
7. Schmidt, K. N., Traenckner, E. B. M., Meier, B., and Baeuerle, P. A. (1995) *J. Biol. Chem.* 270, 27136–27142
8. Schreck, R., Rieber, P., and Baeuerle, P. A. (1991) *EMBO J.* 10, 2247–2258
9. Stamer, J. S. (1984) *Cell* 33, 931–936
10. Nathan, C. (1992) *FASEB J.* 6, 3051–3064
11. Ignarro, L. J. (1991) *Biochem. Pharmacol.* 41, 485–490
12. Morris, B. J. (1995) *J. Biol. Chem.* 270, 24740–24744
13. Gross, R. W., Rudolph, A. E., Wang, J., Sommers, C. D., and Wolf, M. J. (1995) *J. Biol. Chem.* 270, 14555–14588
14. Davidge, S. T., Baker, P. N., McLaughlin, M. K., and Roberts, J. M. (1995) *Circ. Res.* 77, 274–283
15. Lander, H. M., Schajipal, P., Levine, D. M., and Novogrodsky, A. (1993) *J. Immunol.* 150, 1509–1516
16. Lander, H. M., Schajipal, P. K., and Novogrodsky, A. (1993) *J. Immunol.* 151, 7182–7187
17. Lander, H. M., Ogiste, J. S., Pearce, S. F. A., Levi, R., and Novogrodsky, A. (1995) *J. Biol. Chem.* 270, 7017–7020
18. Lander, H. M., Ogiste, J. S., Teng, K. K., and Novogrodsky, A. (1995) *J. Biol. Chem.* 270, 21195–21204
19. Lander, H. M., Milbank, A. J., Tauras, J. M., Hajar, D. P., Hempstead, B. L., Schwartz, G. D., Kraemer, R. T., Mirza, U. A., Chait, B. T., Campbell-Burk, S., and Quilliam, L. A. (1999) *Nature* 391, 380–381
20. Campbell-Burk, S. L., and Carpenter, J. W. (1995) *Methods Enzymol.* 250, 3–13
21. Mirza, U. A., Chait, B. T., and Lander, H. M. (1995) *J. Biol. Chem.* 270, 17185–17188
22. John, J., Schlichting, I., Schiltz, E., Rosch, P., and Wittinghofer, A. (1989) *J. Biol. Chem.* 264, 13086–13092
23. Mistou, M. Y., Jacquet, E., Poulet, P., Rensland, H., Gideon, P., Schlichting, I., Wittinghofer, A., and Parmeggiani, A. (1992) *EMBO J.* 11, 2391–2397
24. Sheffler, L. A., Wink, D. A., Melillo, G., and Cox, G. W. (1995) *J. Immunol.* 155, 866–894
25. Miles, A. M., Bohle, D. S., Glassbrenner, P. A., Hanset, B., Wink, D. A., and Graham, M. B. (1996) *J. Biol. Chem.* 271, 40–47
26. Lander, H. M., Javocina, A. T., Davis, R. J., and Tauras, J. M. (1996) *J. Biol. Chem.* 271, 19705–19709
27. Pai, E. F., Kalbach, W., Kregeel, U., Holmes, K. C., John, J., and Wittinghofer, A. (1989) *Nature* 341, 209–214
28. Scherer, A., John, J., Linke, R., Goody, R. S., Wittinghofer, A., Pai, E. F., and Holmes, K. C. (1989) *J. Mol. Biol.* 206, 257–259
29. Valencia, A., Chardin, P., Wittinghofer, A., and Sander, C. (1991) *Biochemistry* 30, 4675–4649
30. Der, C. J., Pan, B. T., and Cooper, G. M. (1986) *Mol. Cell. Biol.* 6, 3291–3294