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Nitrosopeptide Mapping: A Novel Methodology Reveals *S*-Nitrosylation of Dexras1 on a Single Cysteine Residue

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Summary

***S*-Nitrosylation of specific cysteine residues is a reversible signaling mechanism of nitric oxide (NO) generated by NO synthase (NOS) enzymes. In some proteins, evidence has accumulated that more than one cysteine can be *S*-nitrosylated; however, it is difficult to distinguish *S*-nitrosylation on separate cysteine residues. We report a novel simple, sensitive, and specific procedure for nitrosopeptide mapping. Dexras1 is a monomeric G protein whose guanine nucleotide exchange activity is augmented by NO; the identity and number of its *S*-nitrosylated cysteines is unknown. We describe the radiolabeling of *S*-nitrosylated cysteine residues in Dexras1. A nitrosopeptide map, generated by two-dimensional peptide chromatography, reveals that only a single cysteine is *S*-nitrosylated following NO exposure. Mutagenesis of Cys11 abolished the effect of NO donors on Dexras1, implicating this residue in the NO-mediated activation of Dexras1.**

Introduction

Nitric oxide (NO) is a highly reactive signaling molecule that is made in a wide variety of cells, most prominently: neurons, skeletal muscle, endothelial cells, and certain immune system cells. In these cells, NO is synthesized by one or more of three highly related NO synthase (NOS) isoenzymes, each of which is encoded by a separate gene and named for the initial cell type in which it was isolated. These enzymes, neuronal NOS (nNOS), endothelial NOS (eNOS), and macrophage or inducible NOS (iNOS), synthesize NO from arginine and NADPH in an O_2-dependent reaction. One pathway by which NO signals is by the formation of nitrosothiols [1, 2], a modification of the thiol moiety on cysteine residues, also called *S*-nitrosation or *S*-nitrosylation. The formation of nitrosothiols appears to require the reaction of NO with O_2, resulting in oxidizing NO_x (such as NO_2 and N_2O_3) that serve as the direct oxidizing species that *S*-nitrosylate cysteines [3]. *S*-Nitrosylation can modulate protein function or activity, as was first shown with the *N*-methyl-D-aspartate (NMDA) receptor [4].

The nitrosothiol modification can be detected by heterolytic cleavage of the S-NO bond by $HgCl_2$, followed by colorimetric or fluorometric detection of NO_2^- that forms upon reaction of the released NO^+ and water. Alternatively, the S-NO bond can be cleaved homolytically, usually using a laser, and the released NO· can be detected by a chemiluminescent reaction with ozone [5]. The chemiluminescent method affords higher sensitivity, although the procedure requires specialized equipment.

Nitrosothiol modification of cysteines blocks its ability to react with biotinylmaleimide derivatives, thus allowing the presence of nitrosothiols to be inferred by the reduction in the labeling of protein compared to a non-*S*-nitrosylated control [6]. In practice, it is difficult to detect a slight reduction in the labeling of a *S*-nitrosylated protein compared to its unmodified control if only one or a few of its cysteines is *S*-nitrosylated.

We have described the labeling of *S*-nitrosylated proteins with biotin specifically on *S*-nitrosylated cysteines [7]. Labeling allows *S*-nitrosylated proteins to be readily detected by biotin immunoblotting and allows *S*-nitrosylated proteins to be purified by avidin-affinity chromatography. Labeling is achieved in three steps. In the first step, free thiols in a protein or a protein mixture are blocked by incubation with the thiol-specific methylthiolating agent methyl methanethiosulfonate (MMTS) [8]. Sodium dodecyl sulfate (SDS) is used as a protein denaturant to ensure access of MMTS to buried cysteines. Under the conditions used, MMTS does not react with nitrosothiols [7] or preexisting disulfide bonds [8]. The procedures are performed in the dark to minimize photolysis of the nitrosothiol bonds. In the second step, nitrosothiol bonds are selectively decomposed with ascorbate, which results in the reduction of nitrosothiols to thiols [9, 10]. In the third step, the newly formed thiols are reacted with *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (biotin-HPDP), a sulfhydryl-specific biotinylating reagent [11]. Because MMTS can compete with biotin-HPDP for thiol groups, residual MMTS is removed by desalting on a spin column or by acetone precipitation prior to treatment with ascorbate. By the end of the third step, *S*-nitrosylated proteins are labeled with biotin specifically on cysteine residues that were once *S*-nitrosylated.

This technique has provided insight into the nitrosoproteome. In a screen designed to identify *S*-nitrosylated proteins in the brain [7], several proteins were identified, including the NR2A and NR1 subunits of the NMDA receptor, GAPDH, the sodium-potassium ATPase, actin, and the retinoblastoma gene product (Rb) [7]. Endogenous *S*-nitrosylation of these proteins is abolished in mice harboring a targeted deletion of nNOS [7], establishing that neurally-generated NO mediates the *S*-nitrosylation of these proteins.

Although *S*-nitrosylation appears to be a relatively specific reaction that occurs only on one or a few cys-

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teines within a given protein, mechanisms underlying the selectivity of *S*-nitrosylation have been unclear. By analogy with kinase signaling, a consensus sequence for S-nitrosylation has been proposed, based on the idea that amino acids adjacent to a cysteine may contribute to its ability to be *S*-nitrosylated [12].

Alternatively, *S*-nitrosylation may be facilitated by direct juxtaposition of NOS with a *S*-nitrosylation target. We discovered that nNOS binds selectively to CAPON [13], an adaptor protein that can physically couple nNOS to physiologic targets [14, 15]. CAPON binds to a recently discovered monomeric G protein, Dexras1, resulting in the formation of a ternary complex comprising nNOS, CAPON, and Dexras1. A mechanism for activation of Dexras1 was suggested by analogy with H-ras, a highly similar G protein that is activated by NOS enzymes [16, 17, 18], and can be *S*-nitrosylated [19]. Indeed, Dexras1 is also activated by nNOS [14]. This activation is greatly enhanced by the presence of CAPON and the concomitant formation of the ternary complex [14].

Although H-ras is *S*-nitrosylated on Cys-118 [20], there is no cysteine in the corresponding position of Dexras1. Thus, the *S*-nitrosylated cysteine(s) in Dexras1 are unknown. Proteins vary in the number of cysteines that are *S*-nitrosylated with some, such as the ryanodine receptor, possessing several cysteines that are *S*-nitrosylated [21], whereas caspase-9 and H-ras are *S*-nitrosylated on single cysteine residues [19, 22]. To identify specific cysteines that are *S*-nitrosylated in Dexras1, we have developed an approach analogous to phosphopeptide mapping termed nitrosopeptide mapping. We have modified the biotin-HPDP reagent employed in our *S*-nitrosylation method [7], replacing the biotin with an [^{35}S]-labeled tag. Utilizing this reagent, we demonstrate that Dexras1 is *S*-nitrosylated on a single cysteine. Mutagenesis of cysteine-11 abolishes NO-dependent activation of its guanine nucleotide exchange activity, implicating this residue in NO-dependent activation.

Results

Dexras1 Is *S*-Nitrosylated by Distinct NO Donors

Several lines of evidence suggest that Dexras1 is *S*-nitrosylated by NO. First, like H-Ras, the guanine nucleotide exchange activity of Dexras1 is activated by NO donors [14], as well as by cotransfection of Dexras1 and nNOS in HEK293 cells [14]. NO donors can activate purified Dexras1, and activation of Dexras1 in HEK293 cells by nNOS is not blocked by guanylyl cyclase inhibitors nor is it activated by cell-permeable nonhydrolyzable cGMP analogs [14], ruling out a role for the guanylyl cyclase pathway in the activation of Dexras1.

To determine if Dexras1 is *S*-nitrosylated by NO donors, we incubated 100 ng GST-Dexras1 with 100 μM *S*-nitroso-*N*-acetylpenicillamine (SNAP), a nitrosothiol NO donor, or 100 μM *N*-acetylpenicillamine (NAP), its inactive control. Nitrosylated cysteine residues were biotinylated using the biotin-swap method [7]. SNAP-treated GST-Dexras1 was readily labeled, while biotinylation of NAP-treated GST-Dexras1 was not detectable (Figure 1A). GST is not readily *S*-nitrosylated under these conditions (Figure 1A).

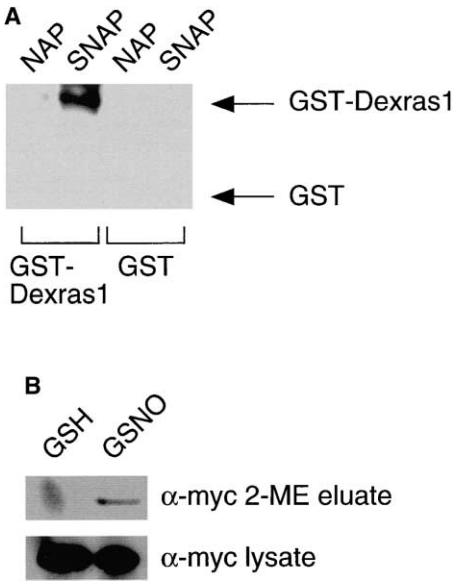

Figure 1. *S*-Nitrosylation of Dexras1 by Different NO Donors

(A) *S* -Nitrosylation of Dexras1 detected by the biotin-swap method. Dexras1 and GST were prepared in *E. coli*, purified by glutathione-affinity chromatography, and then dialyzed to remove free glutathione. Proteins were incubated with 100 μM SNAP, an NO donor, or the control compound NAP. Following the biotin-swap, *S*-Nitrosylation was detected by anti-biotin Western blot. GST-Dexras1 was *S*-nitrosylated, while the control protein GST was not appreciably *S*-nitrosylated under the conditions used.

(B) *S*-nitrosylation of Dexras1 by GSNO in a HEK293 lysate. HEK293 cells were transfected with a plasmid, resulting in the expression of myc-Dexras1. Lysates were treated with 100 μM GSNO or GSH and subjected to the biotin-swap method. Biotinylated proteins were purified on neutravidin agarose and then eluted with 2-ME. Myc-Dexras1 was detected in lysates treated with GSNO, but not lysates treated with GSH. The lower panel shows the expression of myc-Dexras1 in the HEK293 lysates.

S-Nitrosoglutathione (GSNO) is present in tissues at micromolar concentrations and might serve as an endogenous reservoir of NO groups [2, 23]. GSNO is structurally related to SNAP and is also a nitrosothiol NO donor. To determine if Dexras1 can be *S*-nitrosylated by GSNO, we examined the ability of GSNO to *S*-nitrosylate myc-Dexras1 in cellular lysates. HEK293 cells were transfected with a myc-Dexras1-expressing plasmid [14], and lysates were incubated with GSNO or glutathione (GSH). Lysates were then subjected to the biotin-swap method [7, 24], and the biotinylated proteins were purified on neutravidin agarose. The biotin adduct is connected to the protein via a disulfide bond, allowing bound proteins to be eluted from the neutravidin-agarose with 2-mercaptoethanol (2-ME). 2-ME eluates derived from lysates treated with GSNO contained myc-Dexras1 (Figure 1B), while 2-ME eluates from lysates treated with GSH did not contain myc-Dexras, indicating that Dexras1 is *S*-nitrosylated by GSNO in the context of a cellular lysate.

Radioactive Labeling of *S*-Nitrosylated Dexras1

Specific labeling of *S*-nitrosylated cysteines requires the formation of a detectable adduct by reaction with a thiol-

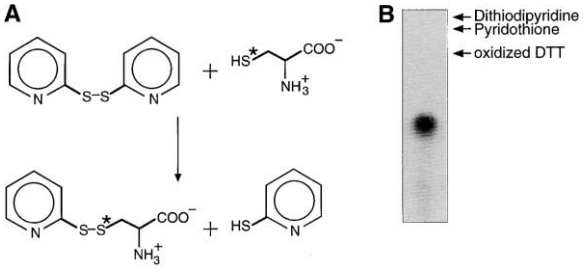

Figure 2. Synthesis and Purification of [^{35}S]APDP

(A) [^{35}S]APDP was prepared by a disulfide exchange reaction between [^{35}S]cysteine and 2,2′-dithiodipyridine.

(B) [^{35}S]APDP thin-layer chromatography indicated essentially complete formation of the product. Unreacted dithiodipyridine, mercaptopyridine, and reduced and oxidized DTT were visualized under UV light and migrated to the positions shown.

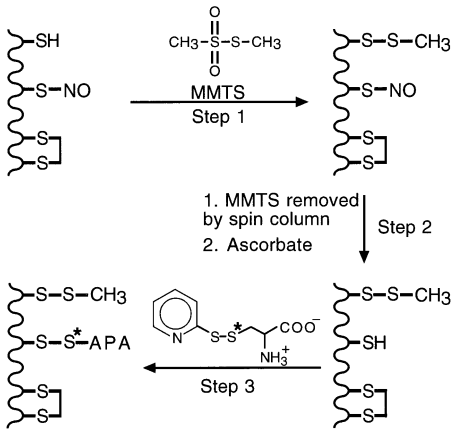

Figure 3. Radiolabeling of *S*-Nitrosylated Proteins

Schematic diagram of radiolabeling procedure. A theoretical protein is indicated with cysteine thiols present in either the thiol, disulfide, and nitrosothiol state. In the first step, MMTS is used to rapidly oxidize thiols, resulting in the essentially complete methylthiolation of cysteines in the protein sample. SDS is used to ensure access of the MMTS reagent to buried thiols. MMTS is removed by precipitation of proteins with acetone and then nitrosothiols are selectively reduced with ascorbate, resulting in the regeneration of cysteines only at residues that were once *S*-nitrosylated. [^{35}S]APDP, an activated mixed disulfide, reacts with these thiols to confer an aminopropionic acid adduct to cysteines. The radioactive isotope is indicated (*).

specific sulfhydryl reagent after reduction of nitrosothiols with ascorbic acid. In our original report, we labeled reduced nitrosocysteines with the activated mixed disulfide compound biotin-HPDP. This compound contains a mixed pyridyl disulfide, a reactive group that is specific for thiols [11]. We developed an analogous compound comprising an identical activated mixed pyridyl disulfide, as well as a radioactive tag in place of the biotin tag used in biotin-HPDP. This compound, [^{35}S]-2-amino-3-(2-pyridyldithio)-propionate (APDP), was chosen for synthesis because it utilizes a precursor, cysteine (2-amino-3-thiopropionic acid), which is readily available in radioactive form. Synthesis of the target compound was achieved by reaction of dithiodipyridine with cysteine (Figure 2A). TLC revealed complete incorporation of [^{35}S]cysteine into the product APDP (Figure 2B).

S-nitroso-Dexras1 was prepared by incubation of the GST-tagged fusion protein with 100 μM GSNO or 100 μM GSH for 30 min. After in vitro *S*-nitrosylation, remaining GSNO or GSH was removed by desalting the protein twice on a spin column. To radiolabel nitrosylated cysteines, Dexras1 fusions were subjected to the biotin-swap assay as before, except [^{35}S]APDP was used in place of biotin-HPDP (Figure 3). In the first step, unmodified thiols in *S*-nitroso-Dexras1 were blocked by the addition of MMTS in the presence of SDS at 50°C for 20 min, conditions that we have previously found to methylthiolate essentially all thiols in a protein sample [7]. Next, protein was precipitated with acetone and resuspended, and nitrosothiols were reduced with ascorbic acid for 1 hr. Lastly, newly formed thiols were reacted with [^{35}S]APDP, resulting in the addition in a [^{35}S] aminothiopropionate adduct on cysteines that were once *S*-nitrosylated (Figure 3). This adduct is a cysteine, resulting in the formation of a cysteine dimer, i.e., cystine, with the ^{35}S radiolabel in the adduct. Because the radioactive tag is connected to the protein via a disulfide, reducing agents (such as 2-ME or DTT) were excluded from all subsequent steps.

Radiolabeled protein was separated from unincorporated [^{35}S]APDP by SDS-PAGE. Protein was transferred to a nitrocellulose membrane, which was then exposed to autoradiography film to locate the radiolabeled protein. Radioactive protein was only detected in samples treated with GSNO, but not GSH (Figure 4A), confirming the specificity of the procedure for *S*-nitrosylated Dexras1.

Nitrosopeptide Mapping of *S*-Nitroso-Dexras1

Inspection of the rat Dexras1 sequence indicates that there is no predicted tryptic peptide from Dexras1 that

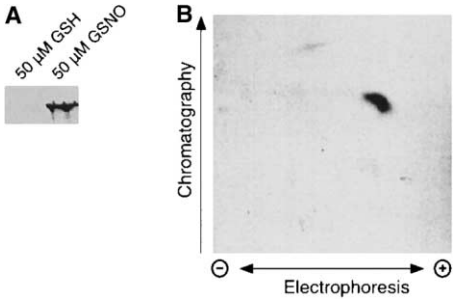

Figure 4. Radiolabeling and Nitrosopeptide Mapping of Dexras1

(A) Radiolabeling of *S*-nitroso-Dexras1. Purified Dexras1 was incubated with GSH or GSNO as indicated and then radiolabeled on modified cysteine residues with [^{35}S]APDP. Only Dexras1 treated with GSNO was detectably labeled by autoradiography.

(B) *S*-nitroso-Dexras1 nitrosopeptide map. After purification of the radiolabeled protein by electroblotting to nitrocellulose and excision, [^{35}S]Dexras1 was trypsinized and tryptic peptides were resolved by two-dimensional peptide mapping. Peptides were subjected to electrophoresis in the *x* axis and ascending chromatography in the *y* axis. Autoradiography revealed a single prominent spot consistent with a single site of *S*-nitrosylation. There are no predicted tryptic peptides from Dexras1 that contain more than one cysteine residue, indicating that only one cysteine is *S*-nitrosylated.

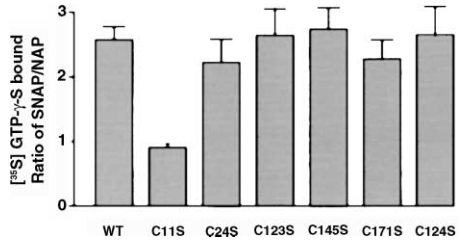

Figure 5. Mutation of Cys11 Prevents NO-Induced Activation of Dexras1

The guanine nucleotide exchange activity of each GST-Dexras1 mutant was measured following incubation with an NO donor, SNAP, and the control compound NAP. The C11S mutant shows no increase in activity following SNAP treatment relative to NAP. Wild-type Dexras1 (WT) and other Cys→Ser mutants showed between 2.0- to 2.8-fold increases in activity following incubation with SNAP. Data are presented as means ±SEM of three to four separate measurements.

would have more than one cysteine in it (S.R.J., M.F., and S.H.S., unpublished data). Thus, the number of spots seen in a peptide mapping experiment would indicate the number of labeled cysteines in Dexras1. To prepare tryptic fragments, the portion of the nitrocellulose membrane containing the protein was subjected to trypsinolysis [25]. The radioactive peptide solution was spotted onto cellulose TLC plates and subjected to electrophoresis in the *x* axis followed by ascending chromatography. Autoradiography of the TLC plate revealed a single major spot suggestive of a single radiolabeled peptide (Figure 4B), indicating that only one cysteine is *S*-nitrosylated in Dexras1.

Identification of Cysteine-11 as the Target of *S*-Nitrosylation in Dexras1

Dexras1 contains seven cysteines of which one, C-276, at the extreme C terminus, is palmitoylated and thus not a likely candidate for *S*-nitrosylation. To identify the *S*-nitrosylated cysteine in Dexras1, we mutagenized the other six cysteines to serine and monitored the guanine nucleotide exchange activity of the mutant GST-Dexras1 fusion proteins. In these experiments, Dexras1 was initially treated with unlabeled GDP to saturate the nucleotide binding sites in Dexras1. After removal of GDP, the proteins were treated with either 50 μM SNAP or NAP for 30 min to activate the protein's intrinsic guanine nucleotide exchange activity. The protein mixture was treated with [^{35}S]GTP-γ-S to label vacant Dexras1 nucleotide binding sites.

As observed previously [14], SNAP treatment of wild-type Dexras1 augmented binding 2- to 3-fold relative to control. Dexras1 mutants containing mutations at cysteines 24, 123, 145, 171, or 224 retained an NO-dependent enhancement of guanine nucleotide exchange (Figure 5). By contrast, augmentation of [^{35}S]GTP-γ-S binding by SNAP was abolished when Cys-11 was mutagenized to serine (Figure 5). Our finding that Cys-11 is required for activation of guanine nucleotide exchange by Dexras1 is consistent with the possibility that Cys-11 is *S*-nitrosylated. Definitive evidence for *S*-nitrosylation of this residue could be obtained by mass spectrometric analysis of Dexras1-derived peptides, as was previously performed to identify the site of *S*-nitrosylation in H-ras [19].

Discussion

Previously, we described a technique for identifying endogenously *S*-nitrosylated proteins [7]. In the present study, we have modified this technique to provide a simple, sensitive, and specific method for nitrosopeptide mapping, enabling investigators to pinpoint a specific amino acid that is *S*-nitrosylated. This method relies on the use of [^{35}S]APDP, a mixed pyridyl disulfide, to specifically modify cysteines that are produced by reduction of nitrosocysteine. In analogy to the heuristic utility of phosphopeptide mapping, the present technique should enhance research into *S*-nitrosylation as a physiologic means of NO signaling by enabling researchers to monitor the *S*-nitrosylation of different cysteines simultaneously. In addition to its use in nitrosopeptide mapping, the [^{35}S]-swap method may have other uses. For example, because the degree of incorporation of ^{35}S can be measured by scintillation counting, the [^{35}S]-swap method may prove useful in experiments in which quantitation of *S*-nitrosylation is desired. We have applied this technique to Dexras1 and determined that a single cysteine is preferentially *S*-nitrosylated in response to NO donors. Mutagenesis experiments implicates Cys11 in the activation of this G protein, suggesting that Dexras1 and H-ras are activated by NO in a different manner.

The finding that only one cysteine in Dexras1 is *S*-nitrosylated is not surprising given the high degree of selectivity in *S*-nitrosylation reactions characterized so far. For example, the NMDA receptor NR2A subunit is *S*-nitrosylated on Cys-399 [26], and caspase-3 is *S*-nitrosylated on Cys-163 [22], although each of these proteins have numerous other cysteine residues. Presumably, the key feature that determines the likelihood of *S*-nitrosylation is the degree of reactivity of the thiol. The reactivity of a cysteine is a function of its nucleophilicity and is reflected by its pK_a. This can be regulated by hydrogen bonding interactions of amino acids adjacent to the cysteine (in either the primary or tertiary protein structure) with the proton in the thiol moiety, resulting in dramatically reduced pK_as and substantially increased nucleophilicity. Indeed, based on *S*-nitrosylation sites previously identified, Stamler and associates have proposed that residues adjacent to cysteines capable of being *S*-nitrosylated form such hydrogen bonding interactions, and they have suggested a possible consensus motif for *S*-nitrosylation [12]. This motif is characterized by hydrophilic residues adjacent to the *S*-nitrosylated cysteine: X-Y-C-Z, where X = G, S, T, C, Y, or N; Y = K, R, H, D, or E; and Z = D or E. In Dexras1, the three residues immediately N-terminal to Cys-11 are Lys-Lys-Met. Conceivably, the lysines contribute to the nucleophilicity of Cys-11. Alternatively, the nucleophilicity of the thiol may be regulated by residues that are brought into proximity to the cysteine as a consequence of the three-dimensional conformation of the protein.

The physical coupling of Dexras1 to nNOS via CAPON may allow Dexras1 to have a lower reactivity towards NO

than other proteins such as H-ras, which is not known to be physically coupled to a NOS protein. In this way, NO-mediated activation of Dexras1 could be limited to cases in which Dexras1 is present in the nNOS ternary complex, reducing the likelihood of spurious activation of unbound Dexras1 by ambient NO or by NO produced in another signaling event and allowing NO signaling to Dexras1 to be compartmentalized.

It is notable that Dexras1 lacks a cysteine corresponding to the *S*-nitrosylated cysteine in H-ras, Cys-118 [20], indicating that a different cysteine must account for the effects of NO on Dexras1. Indeed, Dexras1 is *S*-nitrosylated at Cys-11, which is present in an approximately 40 amino acid-long, N-terminal portion of Dexras1 that is not found in other ras-like proteins including H-ras. Despite the difference in the location of the *S*-nitrosylated residues in these two proteins, both *S*-nitrosylation events activate their respective ras proteins by increasing the dissociation rate of GDP [14, 16]. In the case of Dexras1, the role of the N-terminal domain in which Cys-11 lies is not known, although conceivably *S*-nitrosylation induces a conformational change in the protein, which enhances dissociation of GDP.

Significance

_S_-Nitrosylation is a dynamic modification that may occur on multiple cysteines within a given protein. In studies of protein phosphorylation, phosphopeptide mapping has proved to be an invaluable tool to study the dynamics of phosphorylation at distinct residues. The methodology described here will allow the stoichiometry and dynamics of *S*-nitrosylation to be readily established. The *S*-nitrosylation of Dexras1 on a single cysteine residue identifies a single residue responsible for activation of its guanine nucleotide exchange activity and suggests a role for its N-terminal domain in the regulation of its biological activity.

Experimental Procedures

Synthesis of [^{35}S]-APDP

2-Amino-3-(2-pyridiyldithio)-propionic acid was synthesized by adding 10 μl [^{35}S] cysteine (Perkin-Elmer, Boston, MA, 11 mCi/ml, 1075 Ci/mmol) containing 10 mM DTT (already present in the commercial preparation) to 10 μl of 50 μM 2,2′-dithiodipyridine in methanol. To this solution was added 10 μl phosphate-buffered saline, and this reaction was incubated for 60 min at 25°C. To this reaction was added 10 μl 10:1 methanol:HOAc, and the mixture was spotted onto an analytical silica TLC plate and resolved using 12:6:1:1 ($CHCl_3$:MeOH:HOAc:H_2O). APDP was localized by apposing the TLC plate to X-OMAT Kodak film for 5 s at room temperature and aligning the resulting spot on the film to the TLC plate. APDP migrated to an R_f ~0.4 and was clearly separated from reduced and oxidized DTT, which are included in the commercial cysteine stock, as well as from the unreacted dithiodipyridine and stoichiometrically produced mercaptopyridine. The region of the plate containing the radioactive material was scraped, and the silica was resuspended in 75 μl resuspension buffer (25 mM Hepes (pH 7.7); 0.1 mM EDTA, 0.01 mM neocuproine). After incubation at 25°C for 10 min with frequent vortexing, the silica slurry was clarified by centrifugation. The resulting supernatant contained ~80% of the APDP as determined by scintillation counting.

To verify the identity of [^{35}S]APDP, a nonradioactive APDP standard was prepared. To a solution of 2,2′-dithiodipyridine (8.8 mg, 40 μmol) in 300 μl EtOH were added 500 μl H_2O, 100 μl 1 M HEPES (pH 7.7), and 100 μl cysteine hydrochloride (6.3 mg, 36 μmol). The reaction was allowed to incubate for 15 min at 25°C, at which point the APDP was precipitated by the addition of 9 ml acetone (−20°C). The precipitate was collected by centrifugation and washed two more times in acetone and then dried by rotary evaporation to yield a pale yellow oil. The product was greater than 99% pure by HPLC. MS (ESI) 231.0 [$M + H^+$]. This material comigrated on TLC with [^{35}S]APDP, confirming the identity of the radioactive product.

Biotinylation of *S*-Nitrosylated Dexras1 and H-Ras

The biotin-swap to label *S*-nitrosylated cysteine residues was performed as described previously [7, 24]. In brief, GST-Dexras1 and GST-H-ras were prepared as before [14] and incubated with the indicated concentrations of NO donor or control and then desalted two times on a spin column to remove residual NO donor. The proteins were then incubated with 20 mM MMTS in the presence of SDS, the metal chelators neocuproine and EDTA. After methylthiolation of the protein thiols by MMTS, nitrosothiols were decomposed with ascorbate, and the thiols that formed as a result of ascorbate treatment were labeled with biotin-HPDP (Pierce, Rockford, IL). Biotinylated proteins were detected with a biotin-specific antibody.

For experiments studying myc-Dexras1 in HEK293 lysates, myc-Dexras1 was expressed in HEK293 cells as described previously [14], and cells were sonicated in HEK buffer. Lysates were then centrifuged at 14,000 × g for 10 min at 4°C, and the supernatants were used in subsequent experiments. Lysates (0.8 μg protein/ml) were incubated with the indicated concentrations of GSH or GSNO for 10 min at 25°C in the dark and then subjected to the biotin-swap as described above. Biotinylated proteins were then precipitated with 2 volumes acetone at −20°C for 30 min and then recovered by centrifugation at 14,000 × g for 10 min at 4°C. The pellet was resuspended and incubated with 100 μl neutravidin agarose (Pierce), washed extensively with wash buffer (500 mM NaCl, 1 mM EDTA, 20 mM HEPES [pH 7.7]), and the bound proteins were eluted with 0.1X HEN buffer containing 100 mM 2-ME. The 2-ME eluates were separated by SDS-PAGE and blotted with an anti-myc antibody (Oncogene Sciences) to detect myc-Dexras1.

Radiolabeling Nitrosothiols

GST-Dexras1 was prepared in *E. coli*, bound to glutathione agarose, and eluted with 10 mM glutathione as described [14]. Glutathione was removed by dialysis and then equilibrated in HEN buffer (250 mM HEPES [pH 7.7], 1 mM EDTA, 0.1 mM neocuproine). GST-Dexras1 was incubated with 50 μM GSNO or 50 μM GSH for 1 hr at room temperature in the dark. The GSH or GSNO was removed by passing the mixture through a spin column as above.

Fusion proteins were incubated in HEN buffer to a final concentration of 0.8 μg protein per ml based on the BCA protein assay. To this solution were added four volumes of blocking buffer (9 volumes of HEN buffer plus 1 volume 25% SDS, adjusted to 20 mM MMTS with a 2 M stock prepared in dimethylformamide) at 50°C for 20 min in the dark with frequent vortexing. The MMTS was then removed by precipitation with 2.5 volumes of −20°C acetone for 30 min at −20°C. The protein was recovered by centrifugation at 14,000 × g for 10 min at 4°C. The pellet was resuspended in 50 μl 0.1X HEN buffer containing 1% SDS. To this sample was added 2×10^7 cpm [^{35}S]APDP in a volume of 50 μl and 4 μl of 25 mM sodium ascorbate, and the protein was labeled for 1 hr at 25°C. In trial experiments, further incubation did not result in increased labeling (S.R.J. and S.H.S., unpublished data), suggesting that the reaction was essentially complete, consistent with earlier observations regarding the rapid reaction rates of mixed pyridyldisulfides [11]. After incubation, SDS-PAGE sample buffer was added, and the samples were resolved by SDS-PAGE and transferred to nitrocellulose. Labeled proteins were visualized by autoradiography and processed for nitrosopeptide mapping as described below. All steps preceding [^{35}S]APDP labeling were performed in the dark.

Nitrosopeptide Mapping

[^{35}S]-labeled Dexras1 was separated from unincorporated [^{35}S]APDP by SDS-PAGE. [^{35}S]-Dexras1 was electroblotted onto nitrocellulose (Biorad), and the location of the protein was detected by autoradiography. The region of the nitrocellulose containing Dexras1 was ex-

cised with a razor and placed in a petri dish containing moistened filter paper. Peptides were eluted using a protocol modified from Tempst and coworkers [25]. On a piece of Parafilm was placed 2 μl of digestion solution (100 mM NH_4HCO_3, 0.2% Zwittergent 3-16, 25 ng trypsin [Roche, Indianapolis, IN] per μl) per mm^2 of nitrocellulose, and the nitrocellulose was placed protein-side down on the digestion solution. After incubation in a humidified petri dish at 37°C for 3.5 hr, the membrane was rinsed with 50 μl of H_2O, which was then combined with the remaining peptide hydrolysate, and the pooled fractions were dried down in a Speedvac (Savant, Holbrook, NY). The dried material was resuspended in H_2O and dried in the Speedvac three times to remove residual NH_4HCO_3.

Peptide mapping was performed as described elsewhere [27]. In brief, the dried peptides were resuspended in 10 μl H_2O and then spotted onto a cellulose TLC plate (Kodak 13255) and electrophoresed in (pH 3.4) buffer (19:1:89 acetic acid:pyridine:H_2O) at 500V, using basic fuchsin and phenol red as markers. Ascending chromatography was performed in 15:10:3:12 pyridine:*n*-butanol:acetic acid:H_2O. Film was exposed for 6 days at −80°C, and spots were localized by autoradiography.

Guanine Nucleotide Exchange Assays

[^{35}S]GTP-γ-S binding assays were performed as described earlier [14, 16]. In brief, GST-Dexras1, GST-Dexras1 Cys→Ser mutants, and GST fusions were prepared in *E. coli* as described earlier and remained bound to glutathione agarose for assays. Glutathione agarose beads were added such that a 10 μl of a 50% slurry of beads in storage buffer (20 mM Tris-HCl [pH 7.7], 100 mM NaCl, 1 mM EDTA, 1% Triton X-100) contained 1 μg of protein as determined by Coomassie staining using a bovine serum albumin standard. The slurry (50 μl) was equilibrated in wash buffer (20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$) and then incubated in 1 ml of GDP loading buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, 2 mM GDP) for 30 min at 30°C, washed one time, and then incubated with either 50 μM *S*-nitrosoacetyl penicillamine (SNAP) or the control compound, acetylpenicillamine (Aldrich, Milwaukee, WI) for 30 min at 30°C in the dark, with gentle agitation every 5 min. NO donor was removed by washing two times, and then the slurry was incubated in 50 μl wash buffer to which 1 μCi [^{35}S]GTP-γ-S was added. After 15 min at 30°C, the resins were washed three times with wash buffer, and the bound nucleotides were eluted by heating the samples into 500 μl HENS buffer. The eluted nucleotides were quantitated by scintillation counting.

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