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Original Contribution

Detection of Ras GTPase protein radicals through immuno-spin trapping

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ABSTRACT

Over the past decade immuno-spin trapping (IST) has been used to detect and identify protein radical sites in numerous heme and metalloproteins. To date, however, the technique has had little application toward nonmetalloproteins. In this study, we demonstrate the successful application of IST in a system free of transition metals and present the first conclusive evidence of •NO-mediated protein radical formation in the HRas GTPase. HRas is a nonmetalloprotein that plays a critical role in regulating cell-growth control. Protein radical formation in Ras GTPases has long been suspected of initiating premature release of bound guanine nucleotide. This action results in altered Ras activity both in vitro and in vivo. As described herein, successful application of IST may provide a means for detecting and identifying radical-mediated Ras activation in many different cancers and disease states in which Ras GTPases play an important role.

1. Introduction

Immuno-spin trapping (IST), a technique pioneered by the Mason lab at the National Institute of Environmental Health Sciences, allows for the detection of protein radicals via immunological-based techniques [1]. To date, IST has been limited in its applications and has been used nearly exclusively to detect protein radicals in metalloproteins [1–5]. Recent efforts demonstrate that IST can be used to detect protein radicals in various nonmetalloproteins [6,7]. These studies, however, still required the active site of a separate metalloprotein to generate the free radical oxidizing species used to produce the protein radical on the target protein [6,7]. Herein, we demonstrate the first successful application of IST on a system free of transition metals and present evidence supporting transient protein radical formation in a model Ras GTPase under conditions of nitrosative stress. These findings may alter how we test for protein radicals in nonmetalloproteins and also promote the practical application of IST in proteins in which radical-mediated processes are suspected.

IST is a simple, yet novel, technique that consists of using an oxidizing species, such as nitrogen dioxide ($^{\bullet}NO_2$), to generate a protein radical. The 5,5′-dimethyl-1-pyrroline *N*-oxide (DMPO) spin trap is subsequently used to trap the protein radical intermediate. As DMPO traps the protein radical, a covalently bound DMPO–nitroxide radical adduct is formed. This paramagnetic species can undergo one-electron reduction to the corresponding hydroxylamine or one-electron oxidation to the diamagnetic DMPO–nitrone. Of these redox states, only the nitrone species is thermodynamically stable [1]. The stability of this species permits the use of recently developed anti-DMPO antibodies to detect bound DMPO–nitrone protein adducts using highly sensitive enzyme-linked immunosorbent assays or simple Western blotting [1]. One major advantage IST offers is the ability to detect protein radicals under various experimental conditions on the bench top without the need for an electron spin resonance (ESR) spectrometer. IST can also be conducted at much lower protein concentrations than required for typical spin trapping ESR experiments (μg vs mg quantities). Moreover, as DMPO adduction adds ~111 Da to the molecular weight of the protein, tandem mass spectrometry (MS/MS) approaches can be utilized to unambiguously identify protein radical sites [3–5,8,9]. Perhaps the greatest advantage of immuno-spin trapping is its suitability for detecting protein radical events *in living cells* (i.e., cell cultures and/or in vivo) [10].

Abbreviations: DEA/NO, 2-(*N*,*N*-diethylamino)diazenolate-2-oxide diethylammonium salt; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; eNOS, endothelial nitric oxide synthase; FTICR, Fourier transform ion cyclotron resonance; IST, immuno-spin trapping; •NO, nitric oxide; $^{\bullet}NO_2$, nitrogen dioxide; PI3K, phosphatidylinositol 3-kinase

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The HRas GTPase was chosen for this study for two main reasons. First, numerous studies demonstrate that small free radical oxidants can alter Ras activity both in vitro and in vivo [11–15]. Small free radical oxidants, such as $^{\bullet}NO_2$, are hypothesized to promote guanine nucleotide exchange through generation of a transient Ras thiyl protein radical centered at Cys118 [12–15]. We have suggested elsewhere that electron transfer between the thiyl protein radical and bound guanine nucleotide initiates premature release of the nucleotide. This process can result in exchange of GTP for GDP and activation of the Ras protein in vivo [12–15]. As seen in Fig. 1, the nearest distance between the Cys118 sulfhydryl and the bound GDP is ~7.5 Å, according to the 1CRR NMR structure [16]. Electron transfer over such a distance is common given that a suitable pathway for the transfer exists. Currently, only indirect evidence supports thiyl radical formation of Ras Cys118 in the presence of a free radical oxidant.

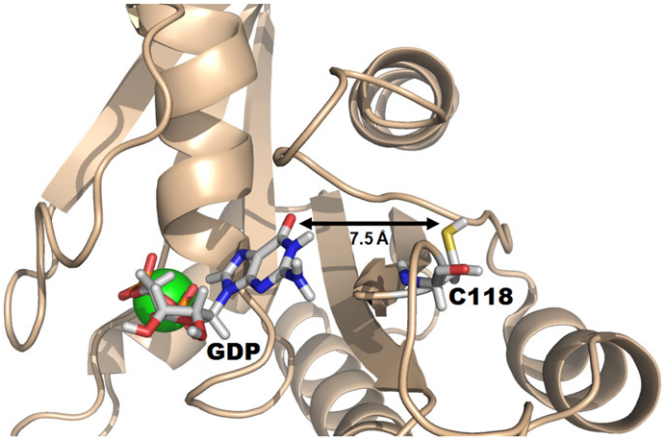

Fig. 1. NMR solution structure (pdb 1CRR) of GDP-bound HRas. Bound GDP and the Cys118 side chain are highlighted in sticks (Mg^{2+} is shown in green). Approximately 7.5 Å separates the bound GDP from the sulfhydryl on Cys118.

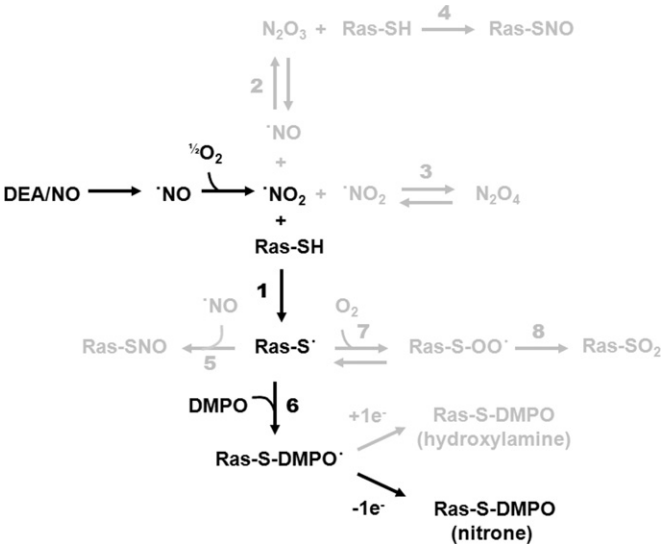

Fig. 2. Ras immuno-spin trapping reaction diagram. The black pathway shows the primary reaction steps involved in $^{\bullet}NO$-mediated Ras immuno-spin trapping experiments. The gray pathways highlight competing reactions associated with the experiment. Reactions and kinetic parameters associated with all reaction steps are shown in Table 1.

Second, Ras GTPases are considered one of the most prevalent oncoproteins in human cancer. Mutations in Ras proteins are present at high levels in pancreatic (~90%), colorectal (35–45%), and lung (~30%) cancers [17]. Recent studies have also linked endogenous nitric oxide ($^{\bullet}NO$), released from active endothelial nitric oxide synthase (eNOS), to enhanced tumor initiation and maintenance in oncogenic Ras-driven pancreatic cancer [18]. Previous in vitro studies from our lab demonstrated that S-nitrosation of Ras at Cys118 does not affect Ras activity [19]. These observations suggest that thiyl radical production at Cys118, rather than Cys118 S-nitrosation, may be a key factor in $^{\bullet}NO$-mediated regulation of Ras activity. We hypothesize that the autoxidation product of $^{\bullet}NO$, $^{\bullet}NO_2$, may contribute to Ras activation during eNOS-enhanced pancreatic tumorigenesis through production of a transient Ras protein radical. Successful detection of the Ras protein radical using IST-based approaches in vitro may lay the groundwork for future tests in cancer cell lysates and/or animal models.

For this study, $^{\bullet}NO_2$ oxidant was generated by autoxidation of $^{\bullet}NO$ liberated from the compound 2-(*N*,*N*-diethylamino)diazenolate-2-oxide diethylammonium salt (DEA/NO). As opposed to bolus addition, the slow release of $^{\bullet}NO$ from DEA/NO is expected to be more representative of cellular $^{\bullet}NO$ production by active eNOS. As shown in Fig. 2 (black pathway) and Table 1, detection of DMPO–nitrone adducts by IST involves a multitude of kinetic steps beginning with the autoxidation of liberated $^{\bullet}NO$ to produce $^{\bullet}NO_2$ and other higher NO oxides [15,20–29]. The slow release of $^{\bullet}NO$ not only simulates active eNOS, but also helps limit formation of the nonradical oxidant dinitrogen trioxide (N_2O_3) [30]. Competing reactions (gray pathways in Fig. 2), unfavorable reaction rates, and low yields of DMPO adduction highlight the challenge of applying IST in nonmetalloproteins. The reactions and associated kinetic parameters for all pathways are listed in Table 1.

As previously stated, IST has traditionally been used to detect protein radicals in heme proteins. The high oxidation potential of the compound I π-cation radical, formed after addition of H_2O_2, drives protein radical formation and DMPO–nitroxide adduction in heme proteins, such as myoglobin [1]. The high-valence oxoferryl ($Fe_{IV}=O$) compound II intermediate then serves as a second oxidizing equivalent to further oxidize the DMPO–nitroxide to the DMPO–nitrone. This zwitterionic DMPO–nitrone species acts as the specific

Table 1
Reaction and kinetic parameters associated with the Ras immuno-spin trapping pathways illustrated in Fig. 2.

Reaction No.	Reaction	k_{for}	k_{rev}	K	Ref.
1	$^{\bullet}NO_2$ + Ras–SH → Ras–S$^{\bullet}$	$\geq 2 \times 10^7$ M^{-1} s^{-1} [a]	n/a	n/a	[15,20]
2	$^{\bullet}NO_2$ + $^{\bullet}NO$ ↔ N_2O_3	1.1×10^9 M^{-1} s^{-1}	4.3×10^6 s^{-1}	2.6×10^2 M^{-1}	[20,21]
3	$^{\bullet}NO_2$ + $^{\bullet}NO_2$ ↔ N_2O_4	4.5×10^8 M^{-1} s^{-1}	—	7×10^4 M^{-1}	[21–23]
4	N_2O_3 + Ras–SH → Ras-SNO	1.6×10^3 M^{-1} s^{-1} [a]	n/a	n/a	[15,24]
5	Ras–S$^{\bullet}$ + $^{\bullet}NO$ → Ras-SNO	3×10^9 M^{-1} s^{-1} [a]	n/a	n/a	[25,26]
6	Ras–S$^{\bullet}$ + DMPO → Ras–S–DMPO$^{\bullet}$	2.6×10^8 M^{-1} s^{-1} [a]	n/a	n/a	[15,27]
7	Ras–S$^{\bullet}$ + O_2 ↔ Ras–SOO$^{\bullet}$	2.2×10^9 M^{-1} s^{-1} [a]	6.2×10^5 s^{-1} [a]	3.3×10^3 M^{-1} [a]	[28,29]
8	Ras–SOO$^{\bullet}$ → Ras–SO_2	2×10^3 s^{-1} [a]	n/a	n/a	[28]

[a] Kinetic rates reported are based on reactions using reduced glutathione.