Structural and Functional Analysis of a Mutant Ras Protein That Is Insensitive to Nitric Oxide Activation†

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ABSTRACT: Ras proteins cycle between active, guanosine triphosphate (GTP)-bound and inactive, guanosine diphospate (GDP)-bound states to mediate signal transduction pathways that promote cell growth and differentiation. It is believed that the major physiological mechanism for Ras activation is via interaction with guanine-nucleotide exchange factors (GEFs). This interaction is highly regulated and results in elevated levels of Ras-GTP by facilitating GDP dissociation. Recently, a novel mechanism of Ras activation has been proposed, whereby nitric oxide (NO) modification of Cys-118, like GEF interaction, populates Ras in its biologically active form by stimulating GDP release. Here, we describe characterization of a variant of Ras, C118S, that is insensitive to NO modification. We have measured the GTPase activity and the GDP dissociation rate of the C118S mutant and found them to be similar to wild-type Ras. We have also analyzed the structure of this mutant using multidimensional heteronuclear NMR methods. Analysis of chemical shifts and distance restraints demonstrates that this mutation has not disrupted the structure of the protein. These results suggest that NO modification of Cys-118 may not alter Ras structure and that the basis of Ras activation by NO is destabilization of a crucial interaction between residues in the GDP-binding pocket and the nucleotide. We have also found that this mutant is a more stable form of Ras at concentrations required for NMR studies, probably due to the removal of a surface-accessible cysteine residue. This stable variant may facilitate structural and biochemical investigations of Ras and other guanine-nucleotide-binding proteins containing a cysteine at this position.

Ras proteins are small guanine-nucleotide binding proteins that function as highly regulated molecular switches (Bourne et al., 1991). They cycle between GTP-[1] and GDP-bound forms to mediate signal transduction pathways leading to a diverse array of biological responses such as cell growth, cell differentiation, and programmed cell death. The best-characterized Ras-mediated signal transduction pathway involves the activation of receptor tyrosine kinases [reviewed in Khosravi-Far and Der (1994)]. Stimulation of a membrane-bound receptor leads to the activation of GEFs. These bind to Ras, promoting the dissociation of GDP, and Ras becomes populated in its activated GTP-bound form due to the excess of GTP in the cell. Once GTP-bound, Ras can interact with its downstream target, the Raf-1 serine/threonine kinase, to stimulate a kinase cascade that leads to transcriptional activation of genes that regulate the growth and differentiation state of cells (Crews & Erikson, 1993; Avruch et al., 1994; Davis, 1993). Signal transmission is down-regulated by the action of GAPs, which stimulate the intrinsic GTPase of Ras to produce the inactive GDP-bound form (Polakis & McCormick, 1993). The activity of Ras is tightly controlled by proteins that regulate the state of its bound guanine-nucleotide. The consequences of its inappropriate activation are apparent, since oncogenic forms of Ras have been identified in approximately 30% of human tumors.

The free radical nitric oxide (NO) plays an important role in redox regulation and has been implicated in intracellular signaling [see Stamler (1994) for a review]. It exerts its effects by nitrosylating nucleophiles, such as cysteine thiol groups, tyrosine hydroxyl groups, and metal centers. Recently, it has been shown that guanine-nucleotide binding proteins, particularly Ras, are targets of NO in the cell (Lander et al., 1993, 1995a) through a single nitrosylation event. NO decreases the overall affinity of Ras for guanine-nucleotides (Lander et al., 1995b), leading to an enhanced rate of guanine-nucleotide exchange and consequently an increased population of Ras-GTP. Thus, it is possible that NO stimulation of guanine-nucleotide exchange is an alternative physiological mechanism for the activation of signal transduction pathways mediated by Ras that are sensitive to redox stress (Lander et al., 1996a).

NO modification of Ras can be monitored by mass spectrometry (Mirza et al., 1995). A truncated form of Ras lacking 23 residues from the C-terminus has identical activity to the full-length protein (John et al., 1989) and contains three Cys residues. Cleavage of the truncated form of Ras with CNBr yields three fragments, each containing one Cys residue, allowing the NO-modified Cys residue to be localized to Cys-118 (Lander et al., 1996b). The identification of this Cys was confirmed by analysis of a mutant form of Ras in which this residue was changed to a Ser. This mutant was not modified by NO, as judged by mass spectrometry, and its GTPase activity was not stimulated by NO *in vitro.* Furthermore, the C118S mutant was unrespon-

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[1] Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; GAP, GTPase activating protein; GDP, guanosine diphosphate; GEF, guanine-nucleotide exchange factor; GTP, guanosine triphosphate; HSQC, heteronuclear single quantum correlation; NF1-GAP, GTPase activating domain of neurofibromin 1; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy.

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sive to NO *in vivo*. Accessibility calculations using a solvent probe of radius 1.4 Å show that the side-chain of Cys-118 is approximately 10 000 times more accessible than either Cys-51 or Cys-80 in a typical Ras-GDP NMR structure (Kraulis et al., 1994). These observations are consistent with studies conducted by Hata-Tanaka et al. (1989), where Cys-118 was the only cysteine residue in truncated Ras that was modified by a maleimide spin label (Hata-Tanaka et al., 1989).

It is clear from the above discussion that NO modification of Ras has the same outcome as binding of GEFs to Ras, since both of these interactions lead to enhanced GDP release, increased population of Ras-GTP, and the subsequent stimulation of multiple signal transduction pathways. The mechanisms of both NO-stimulated and GEF-stimulated GDP release are poorly understood. Mutagenesis of residues 28, 117, 119, 146, and 156 leads to enhanced GDP dissociation and interferes with GEF regulation (Reinstein et al., 1991; Der et al., 1988; Hwang et al., 1993; Feig & Cooper, 1988; Quilliam et al., 1995). It is thought that GEFs work by destabilizing the GDP-bound form of Ras, possibly by interfering with residues of Ras that form crucial contacts with GDP. The basic mechanism of Ras activation by NO is likely to show some similarity to that of GEFs, since its binding also destabilizes the GDP-bound form. Modification of C118 with NO disrupts interactions between the Ras protein and its bound nucleotide, leading to enhanced GDP release.

In this study, we have examined the structure of C118S Ras using heteronuclear NMR to determine whether the perturbation of residue 118 by mutation leads to structural alterations around the guanine-nucleotide binding site. In addition, both the guanine-nucleotide exchange and the GTPase activity of the mutant have been assessed to investigate whether there are any other biochemical effects of the mutation apart from a deficiency in NO binding. We have also examined whether the properties of this mutant make it more favorable for structural analysis.

MATERIALS AND METHODS

Protein Production and Purification. The C118S truncated (1-166) Ras protein was expressed and purified as described previously (Campbell-Burk & Carpenter, 1995). NMR samples were prepared as before (Campbell-Burk et al., 1992; Kraulis et al., 1994).

Phosphate Release Assay. Ras (400 ng) was incubated in labeling buffer (20 mM Tris, pH 8.0, 1 mM DTT, 200 mM ammonium sulfate, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mg/mL BSA, and 20 μCi [γ-^{32}P]GTP) for 5 min at room temperature. The labeling reaction (total volume = 100 μL) was stopped by adding 1 μL of 0.5 M $MgCl_2$, mixing and placing on ice. Free [γ-^{32}P]GTP was separated from labeled Ras by passing the reaction mixture through a 0.8 × 16 cm G-25 Sephadex (Pharmacia) column which had been equilibrated with GAP buffer (20 mM Tris, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, and 1 mg/mL BSA). Fractions (0.5 mL) were collected and analyzed with a Geiger counter to determine which fractions contained the largest amount of labeled Ras. The highest concentration of Ras in a single fraction was 20 nM. From this fraction, 60 μL was mixed with 240 μL of GAP buffer containing 0.06 pmol of the NF1-GAP to obtain final concentrations of 4 nM Ras and 0.2 nM NF1-GAP. The reaction mixture was incubated for 0, 5, 10, or 20 min. At the end of each time point, a 20 μL aliquot was removed (in triplicate) and applied to a nitrocellulose filter with a 0.1 μm pore size (Schleicher and Schuell) under vacuum filtration. The filter was washed 4 times with 2 mL of wash buffer (20 mM Tris, pH 8.0, 100 mM NaCl, and 30 mM $MgCl_2$). The filters were then put in vials containing 8 mL of ScintiSafe Econo 2 (Fisher) scintillation fluid and counted for ^{32}P.

Guanine Nucleotide Exchange Assay. Ras (400 ng) was incubated in labeling buffer, as described above, for 5 min at room temperature. The labeling reaction (total volume = 100 μL) was stopped by adding 1 μL of 0.5 M $MgCl_2$, mixing and placing on ice. The labeled Ras (75 μL) was put in 300 μL of exchange buffer (final concentration 4nM Ras, 20 mM Tris, pH 8.0, 1 mM DTT, 5 mM $MgCl_2$, 1 mg/mL BSA, 500 μM GTP), mixed, and incubated for 0, 5, 10, 20, or 30 min at room temperature. At each time point a 20 μL aliquot was removed (in triplicate), transferred to nitrocellulose as above, and the filters counted for ^{32}P.

NMR Spectroscopy. NMR experiments were recorded on a Bruker AMX spectrometer operating at 500 MHz for protons. A 3D ^{15}N-edited NOESY experiment with a mixing time of 100 ms was recorded with gradient artifact suppression and preservation of water magnetization using flip-back pulses (Grzesiek & Bax, 1993; Stonehouse et al., 1994). The 3D NOESY was recorded as 512 × 120 × 32 complex points, with spectral widths of 7042.254, 7042.254, and 1000 Hz in the f3, f1, and f2 dimensions, respectively. Spectra were processed using *Felix95* (Biosym Tech. Inc.) and analyzed using *XEASY* (Bartels et al., 1995). NMR data for the C118S mutant were compared to a 3D ^{15}N-edited NOESY that had been recorded previously on wild-type Ras (Kraulis et al., 1994).

RESULTS

Phosphate Release and Guanine Nucleotide Exchange Assays. In order to assess whether the C118S mutation perturbed the intrinsic biochemical and regulatory properties of Ras, we compared the GTPase activity in the presence and absence of NF1-GAP as well as the intrinsic GDP dissociation rate of both wild type Ras and the C118S variant. As shown in Figure 1, the GTPase activities of both the C118S mutant and wild-type Ras were stimulated by NF1-GAP. By contrast, a G60A mutant of Ras, previously shown to possess deficient GTPase activity (Hwang et al., 1996), was not stimulated by NF1. The data for unstimulated GTPase activity for each form of Ras are not shown, but the curves are essentially the same as those shown in Figure 1 for the G60A mutant. These results indicate that the C118S mutation does not perturb either the intrinsic or GAP-stimulated GTPase activity of Ras.

We have also measured the intrinsic rates of exchange of the C118S mutant of Ras, since the modification of C118 by NO is responsible for changing the rate of guanine-nucleotide exchange. The rate of ^{32}P-GTP release upon incubation of Ras-[γ-^{32}P]GTP with excess unlabeled GTP was measured as a function of time for both wild-type and C118S Ras. Inspection of Figure 2 shows that the rates of exchange of wild-type and C118S mutant Ras are essentially the same. Consistent with our observations that the intrinsic and GAP-stimulated activity of Ras does not appear to be

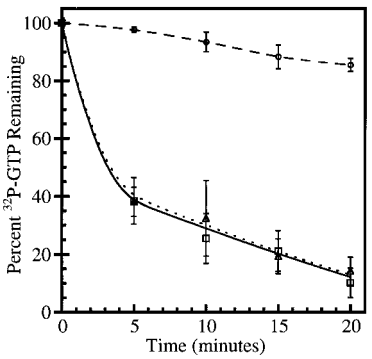

FIGURE 1: Results of the GAP-stimulated GTPase assay: solid line, wild-type Ras; dotted line, C118S mutant; dashed line, G60A mutant. The assay monitors the amount of ^{32}P-GTP remaining as a percentage of the starting concentration over the course of the reaction. The errors shown are the standard error in the mean, calculated by dividing the standard deviation by $(n)^{1/2}$, where *n* is the number of samples at each point. G60A is included as a negative control. This is a mutant form of Ras which is deficient in GAP-stimulated GTP hydrolysis (Hwang et al., 1996). The curve shown for the G60A mutant is essentially the same as the unstimulated rate of GTP hydrolysis.

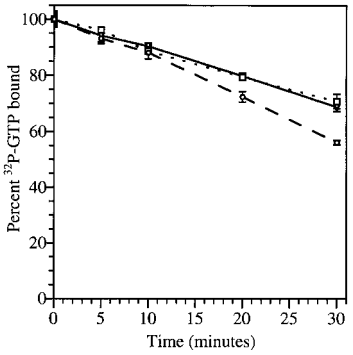

FIGURE 2: Results of the intrinsic GDP dissociation assay: solid line, wild-type Ras; dotted line, C118S mutant; dashed line, G60A mutant. The error bars were generated as described for Figure 1.

altered by the C118S mutation, the apparent rate of GDP dissociation also appears unaffected by this mutation.

The major function of Ras is to cycle between GDP- and GTP-bound forms. The results of the assays we have performed suggest that the C118S mutant of Ras is fully capable of this function.

NMR Analysis. Multidimensional NMR methods were utilized to determine whether there were any structural perturbations in the C118S mutant. It was possible to assign fully the 3D ^{15}N-edited NOESY of this C118S Ras mutant based upon a comparison with the previously assigned wild-type protein (Campbell-Burk et al., 1992; Kraulis et al., 1994). As Figure 3 shows, the positions of the resonances in the HSQC spectra are very similar. A detailed analysis of the chemical shifts of the backbone amide resonances in the wild-type and mutant spectra, as shown in the top of Figure 4, reveals that the chemical shift changes are localized to residue 118 and residues that are close to the site of the mutation in the 3D structure. Apart from the changes in Cys/Ser-118 and Asp-119, there are chemical shift alterations in His-27, Thr-144, Ser-145, and Arg-149. Amide groups are particularly sensitive to slight changes in pH and temperature, so it is possible that their resonances may exhibit chemical shift changes due to these effects. Thus, we have also examined the chemical shifts of both the $C^{\alpha}H$ and $C^{\beta}H$ resonances, and these are shown in the lower half of Figure 4. These resonances could be unambiguously assigned from the 3D ^{15}N-edited NOESY for more than 90% of the residues. The chemical shift changes for these aliphatic protons are also localized to Cys/Ser-118, and the area around Glu-143 and Thr-144 and are in agreement with the observations made for the amide chemical shift changes with the exception that His-27, which had a large ^{15}N chemical shift change but no changes in amide proton $C^{\alpha}H$ or $C^{\beta}H$ shifts. This and the absence of any other changes in the vicinity of this residue suggest that the change in His-27 is due to a slight change in sample pH rather than the mutation.

Although chemical shifts can be a sensitive probe of structural changes, it is also possible that changes in the structure may occur without perturbing the position of the resonances. Thus, we undertook a detailed analysis of the NOE cross-peaks that were observed in the 3D ^{15}N-edited NOESY and compared these to the NOE cross-peaks that had been assigned in the wild-type Ras spectra. In all, we compared the strengths of approximately 350 NOEs which had originated from 57 amide protons. Essentially we could not detect any differences between the presence or intensity of NOEs between the wild-type and mutant proteins. These NOEs included short-, medium-, and long-range interactions. Thus, it appears that there are no structural changes in this mutant form of Ras compared to wild-type. This result is not surprising, since both GDP dissociation and the intrinsic and GAP-stimulated GTPase activity were unaltered.

DISCUSSION

Examination of the three-dimensional structure of Ras-GDP determined by NMR (Kraulis et al., 1994) and crystallographic [see, for example, Wittinghofer and Pai (1991) and Milburn et al. (1990)] methods reveals that the residues which experience significant chemical shift perturbations in the C118S mutant (residues 143, 144) are close in the 3D structure to residue 118, as shown in Figure 5. These chemical shift differences most likely reflect a change in the chemical environment rather than a structural change, since there were no significant NOE differences between wild-type Ras and the C118S variant. The residues influenced by the mutation are located in two loops (L8 and L10) which consist of highly conserved stretches of amino acids involved in binding the guanine ring.

The residues around Cys-118 form the NKXD motif which is conserved in all Ras and Ras-related proteins, elongation factors, and heterotrimeric G-proteins [reviewed in Kjeldgaard et al. (1996)]. The conservation of these residues reflects the roles of their side-chains in both binding interactions and the specificity of guanine-nucleotide recognition: Asn-116 interacts with both the hydroxyl group of Thr-144 and with the N7 atom of the guanine ring; Lys-117 interacts with the oxygen of the ribose ring and Asp-119 interacts with the hydroxyl group of Ser-145 and with the NH and NH_2 groups in the guanine ring. Cys-118 in Ras correponds to the most variable in this motif (X). Residues 116, 117, 119, and 146 contribute to the high-affinity binding of GDP for Ras, since their mutation leads to increased rates of GDP dissociation and thus oncogenic activation (Der et al. 1988; Feig and Cooper, 1988). Furthermore, mutation of Asn-116 and Lys-117 can disrupt interactions with GEFs (Hwang et al., 1993). It is thus likely that the mechanism of NO activation of Ras interferes with one or more of these contacts.

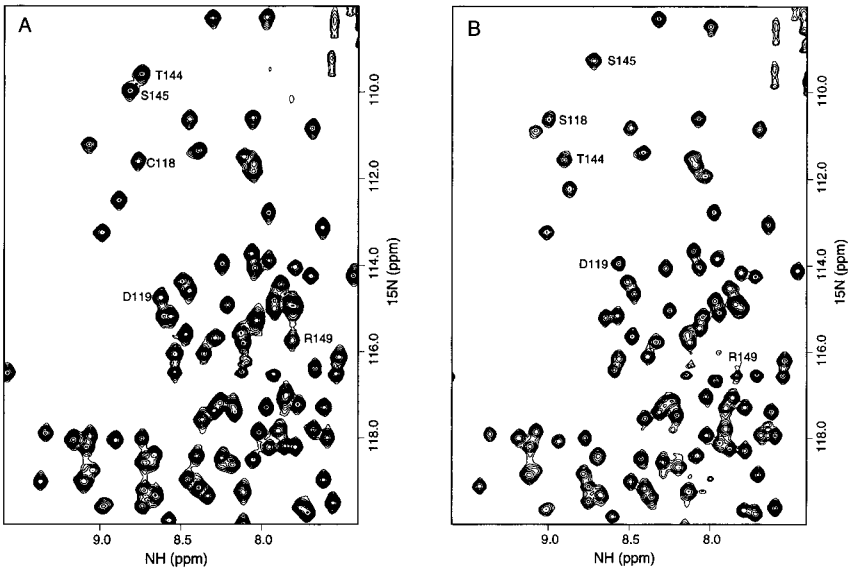

FIGURE 3: Comparison of a portion of the ^{1}H-^{15}N HSQC spectra of (A) wild-type and (B) C118S mutant. The resonances which are in significantly different positions in the two spectra are labeled with the assignments.

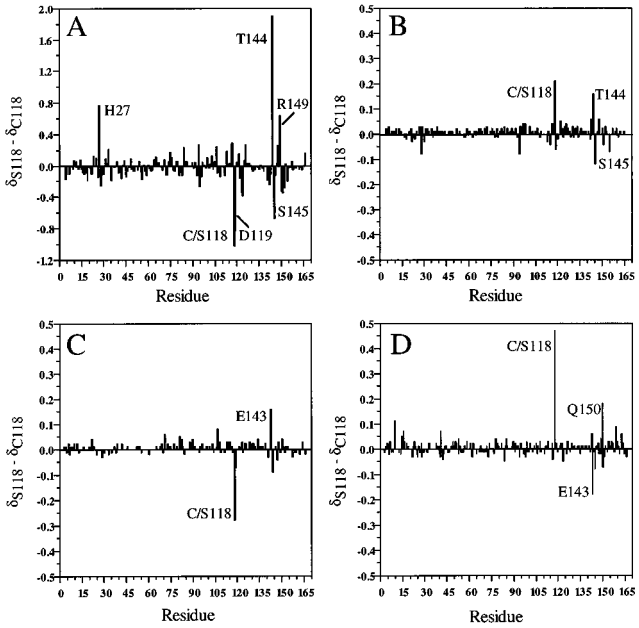

FIGURE 4: Comparisons of the chemical shifts of wild-type and C118S mutant 1-166 Ras. In each case the vertical axis shows the chemical shifts in the wild-type spectra subtracted from those in the C118S spectra. (A) ^{15}N chemical shifts measured from HSQC spectra. Note the different vertical scale used in this bar chart compared to the other three. (B) Backbone amide chemical shift differences measured from HSQC spectra. (C) $C^{\alpha}H$ chemical shift differences measured from 3D ^{15}N-edited NOESY spectra. (D) $C^{\beta}H$ chemical shift differences measured from 3D ^{15}N-edited NOESY spectra.

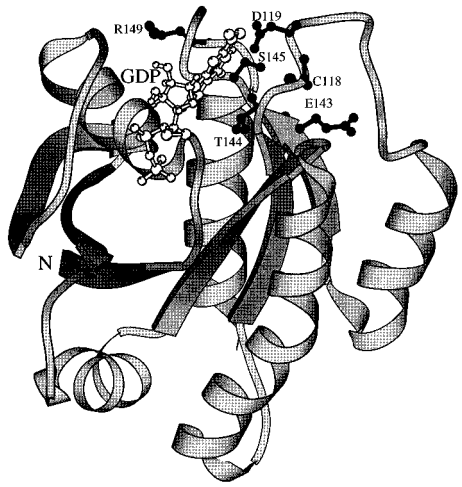

FIGURE 5: A Molscript (Kraulis, 1991) representation of a typical NMR structure of Ras-GDP (Kraulis, 1994). The GDP is shown in white. The side-chains of the residues whose chemical shifts are significantly perturbed in the C118S mutant are shown as ball-and-stick representations in black.

In summary, we have shown that the Ras C118S mutant, which is insensitive to NO activation, is structurally and functionally very similar to wild-type Ras. All of the changes that we have observed by NMR suggest that the C118S mutation causes a small, localized perturbation in the chemical environment of residues 118, 143, and 144, which can be explained by the substitution of a Cys residue with a Ser. Consistent with our structural results, this substitution does not affect the apparent GDP-dissociation rate or the intrinsic or GAP-stimulated GTPase activities. Hence, even though modification of this Cys residue by NO results in an increased rate of guanine-nucleotide exchange, mutation of this residue to Ser does not affect GDP/GTP cycling. This suggests that Cys-118 does not interact directly with GDP or form crucial interactions with other residues involved in binding the guanine-nucleotide. This is consistent with the lack of sequence conservation of this residue in Ras and Ras-like proteins [reviewed in Chardin (1993), Kjeldgaard et al. (1996)], as it can tolerate several amino acid substitutions, such as Ser, Lys, Leu, and Val. The lack of structural and biochemical differences between Cys-118 and Ser-118 forms of Ras suggests that the mechanism of NO activation of Ras is not due to the disruption of interactions between Cys-118 and neighboring residues, but rather may involve disruption of another crucial interaction, with the result that GDP dissociation is enhanced. It is possible that nitrosylation of 118 does not alter the structure, but rather the polar *S*-nitrosothiol group may compete with interactions between the protein and the bound nucleotide, weakening them. This potential mechanism of NO-mediated Ras activation is currently under investigation in our lab.

It is of interest to compare the sequences of other guanine-nucleotide binding proteins in this region to determine their sensitivity to NO activation. As mentioned above, within the NKXD motif, X is always Cys in the mammalian Ras proteins. Comparison with the sequences of other guanine-nucleotide binding proteins shows that Rab1, Rab3, Rab8, Rap1a, and Rap1b all have Cys in this position, and thus they could be activated by NO in a similar manner. In addition, the G-domain of EF-Tu also has a cysteine in this position, suggesting that NO can also bind (and presumably activate) these proteins. The α subunits of the heterotrimeric G proteins and the Rho, Rac, Ran, and Ral family do not have Cys in this position, suggesting that either NO does not activate these proteins or it does not do so by the same mechanism.

Our experience with truncated, wild-type Ras NMR samples has suggested that they are not stable over a long period of time and appear to aggregate, forming precipitates at the air interface. One mechanism by which this sample aggregation may occur is through intermolecular oxidation mediated through solvent-exposed Cys residues. As we have mentioned above, accessibility calculations suggest that Cys-118 is the most solvent-exposed of the three free Cys residues in Ras (Lander et al., 1996). This has led us to believe that this residue is a potential site of oxidation and may be involved in the formation of intermolecular disulfide bonds. It appeared that the Cys-118 to Ser mutant did not aggregate as quickly as wild-type Ras at the high concentrations required for NMR studies and that it was stable for several months. T2 experiments recorded after 3 months were essentially identical, suggesting that the protein was still monomeric (data not shown). Since Ras is a potential drug target, a more stable form, whose biochemical properties and structure are the same as that of the wild-type protein, will be useful for all studies in the future.

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