Redox regulation of cell signalling

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SIR — We have been investigating the structural basis of the interaction between nitric oxide (NO) and the *ras* oncogene product p21 to gain insight into how redox signalling is achieved in cells. Our approach is one way to address the more general problem of finding a molecular target for redox-active environmental toxicants and free radicals. We report here that we have identified the site of molecular interaction between NO and $p21^{ras}$ that is responsible for the initiation of signal transduction.

In our earlier studies we found that a single *S*-nitrosylation event on full-length $p21^{ras}$ produced enhanced guanine nucleotide exchange[1]. Our present *in vitro* studies used $p21^{ras}$ lacking the carboxy-terminal 23 amino acids, as this form of the protein has identical biochemical activity to the wild-type enzyme[2]. To identify the exact site of *S*-nitrosylation, we cleaved $p21^{ras}$ with cyanogen bromide, which yielded three fragments, each containing one cysteine residue. Fragment 1, containing Cys 51, has a relative molecular mass (M_r) of 7,203; fragment 2, containing Cys 80, has M_r 4,540; and fragment 3, containing Cys 118, has M_r 6,225.

We monitored each of the Cys residues for *S*-nitrosylation by subjecting the digested mixture to analysis using electrospray-ionization mass spectrometry (ESI-MS)[3].

As Fig. 1*a* shows, CNBr digestion of p21^{ras} yields a fragment with M_r 6,223±2, corresponding to fragment 3 (containing Cys 118). Treatment of p21^{ras} with NO and subsequent cleavage with CNBr produces a fragment 3 with a new mass clearly indicative of *S*-nitrosylation[3] (an increase of 29±1; Fig. 1*a*). We did not find fragments 1 and 2.

To confirm that Cys 118 is indeed the site of *S*-nitrosylation, we generated a form of p21^{ras} identical to the wild-type enzyme, except that Cys 118 was changed to a Ser residue (referred to as p21^{ras}C118S). This modification only changes the sulphur atom of Cys 118 to oxygen, reducing the M_r of the enzyme by 16, to 18,836. We treated wild-type p21^{ras} with NO under conditions which produced approximately 50% *S*-nitrosylation. Analysis by ESI-MS reveals the parent enzyme (M_r 18,852) and a singly *S*-nitrosylated derivative (M_r 18,882; Fig. 1*b*, blue line). Treatment of p21^{ras}C118S with NO under identical conditions gives no *S*-nitrosylated product, but only the parent enzyme (Fig. 1*b*, red line). These data identify Cys 118 as the molecular target of NO on p21^{ras}.

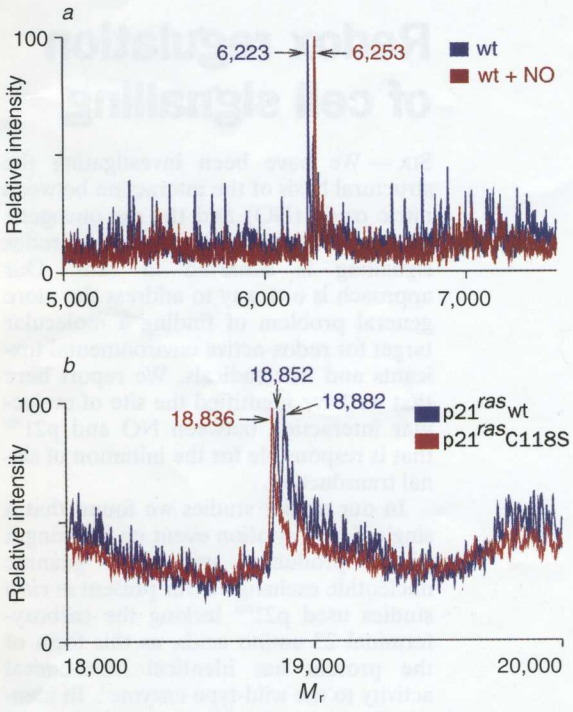

FIG. 1*a*, ESI-MS analysis of CNBr-digested p21^{ras}. Wild-type (wt) p21^{ras}, either untreated (blue line) or treated with equimolar NO (red line), was subjected to CNBr digestion and ESI-MS. Representative data from 4 experiments are shown. *b*, ESI-MS analysis of NO-treated p21^{ras} and p21^{ras}C118S. Wild-type p21^{ras} (blue line) or p21^{ras}C118S (red line) was treated with equimolar NO for 10 min before analysis by ESI-MS. Representative data from 3 experiments are shown.

We next examined whether NO could induce nucleotide exchange on GDP-preloaded p21^{ras}C118S *in vitro*. NO (25 μM) stimulates [γ-^{32}P]GTP hydrolysis of GDP-preloaded wild-type p21^{ras} 9.34±0.98-fold (n=6). In contrast, NO (25 μM) has no effect on the exchange rate of p21^{ras}C118S (0.98±0.36-fold; n=6). Using Jurkat T cells mock-transfected or stably transfected with an expression plasmid encoding a full-length version of p21^{ras}C118S (residues 1–189), we examined the ability of NO to activate the kinase activity of mitogen-activated proteins (MAPs). Immunoprecipitation of the MAP kinases ERK1 and ERK2, from wild-type cells treated with the NO-generating compounds *S*-nitroso-*N*-acetylpenicillamine (300 μM) or sodium nitroprusside (1 mM), results in enhanced phosphorylation of the ERK substrate (163±8 and 229±12% of control, respectively; n=4). In contrast, Jurkat T cells stably expressing p21^{ras}C118S (1–189) do not respond to these compounds in this *in vitro* kinase assay (104±6 and 106±3% of control; n=4), although the combination of phorbol myristate acetate (100 ng ml^{-1}) and the calcium ionophore A23187 (500 ng ml^{-1}) stimulates MAP kinase activity in both cell types (221±8 versus 261±9% of control, parental versus transfected; n=3).

The crystal structure of p21^{ras} is well defined[4,5]; modelling studies show that, of the three Cys residues in our p21^{ras} preparation, Cys 118 is most exposed to the solvent (Fig. 2). We calculated the solvent-accessible surface of p21^{ras} complexed to GDP. Whereas the Cys 51 and Cys 80 side chains are fairly well shielded from the solvent, Cys 118 is exposed. The fact that residues Cys 80 and Cys 51 are so buried provides a structural explanation for the single *S*-nitrosylation that occurs on p21^{ras} on exposure to NO-related species.

These data indicate that Cys 118 is a critical site of redox regulation of p21^{ras}, and that *S*-nitrosylation of this residue triggers guanine nucleotide exchange and downstream signalling. Thus, signalling to the nucleus by redox-active species may involve triggering of a molecular switch on p21^{ras}.

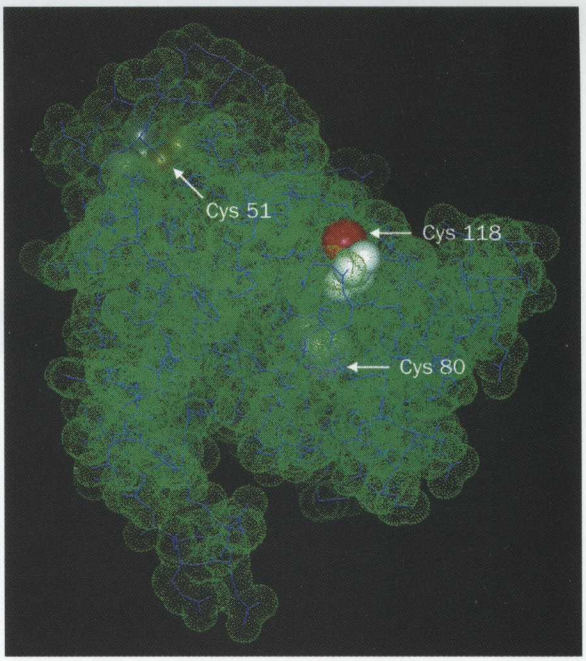

FIG. 2 Surface nature of Cys 118 in the crystal structure of the p21^{ras} preparation we studied. Cys side chains (arrowed) are red (Cys 118), purple (Cys 80) and yellow (Cys 51); Cys backbones are white.

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