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Role of cysteine$_{62}$ in DNA recognition by the P50 subunit of NF-$\varkappa$B

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ABSTRACT

A powerful chemical modification procedure has been developed to define determinants of DNA recognition by the p50 subunit of NF-$\varkappa$B. Differential labelling with [^{14}C] iodoacetate has identified a conserved cysteine residue, Cys62, that was protected from modification by the presence of an oligonucleotide containing the specific recognition site of the protein. To determine the importance of this cysteine residue, each of the conserved cysteines in p50 was changed to serine and the DNA binding properties of the mutant proteins determined. Scatchard analysis indicated that the C62S mutant bound to its DNA recognition site with a 10-fold larger dissociation constant than the wild type protein, while the other two mutants bound with an intermediate affinity. Dissociation rate constant measurements correlated well with the dissociation constants for the wild type, C119S, and C273S p50 proteins, whereas the p50 C62S-DNA complex dissociated anomalously quickly. Competition analyses with oligonucleotide variants of the DNA recognition site and nonspecific *E.coli* DNA revealed that the C62S p50 mutant had an altered DNA binding site specificity and was impaired in its ability to discriminate between specific and non-specific DNA. Thus the sulphydryl group of Cys62 is an important determinant of DNA recognition by the p50 subunit of NF-$\varkappa$B.

INTRODUCTION

One seemingly ubiquitous DNA binding protein involved in the expression of a number of both viral and cellular genes is the mammalian transcription factor NF-$\varkappa$B (1). NF-$\varkappa$B has been shown to comprise two polypeptides of 50 kD (p50) and 65 kD (p65) molecular weight (2, 3). Each subunit makes a contribution to the binding site specificity of the p50-p65 NF-$\varkappa$B heterodimer (4) which makes base and backbone contacts over the 10 base pair NF-$\varkappa$B recognition site (5, 6). Both the p50 and p65 polypeptides posess a highly conserved N-terminal region responsible for DNA binding and dimerisation (7–10)—such that the p50 homodimer DNA binding specificity is similar to that of the p50-p65 heterodimer (3, 4). The highly conserved DNA binding and dimerisation N-terminal regions of p50 and p65 are also shared by a number of other proteins including the 59 kD avian viral oncoprotein v-*rel* (11) and its avian and mammalian cellular c-*rel* counterparts (12), by a c-*rel* -related protein whose gene is transcribed in early *Xenopus laevis* embryos (13), and by the *Drosophila* maternal effect morphogen dorsal (14, 15). More recently characterised members of this family include the 60 kD RelB protein (16), p50B (17) and p49, the latter two being derived from larger precursors (18).

The high degree of homology between the DNA binding domains of the rel family members and the similar DNA binding specificity of the NF-$\varkappa$B p50-p65 heterodimer and the p50 homodimer allowed the use of the DNA binding and dimerisation region of p50 as a simplified system to study the determinants of specific DNA recognition by NF-$\varkappa$B. Although transfection experiments (18, 19) have suggested that the c-rel, p65, and p49 proteins, but not the p50 subunit of NF-$\varkappa$B, could activate transcription from reporter genes containing $\varkappa$B binding sites in their promoters, recent experiments using over-expressed NF-$\varkappa$B subunits have shown that homodimers of the p50 subunit can activate transcription *in vitro* (20, 21).

It has been previously demonstrated that the DNA binding activity of the *fos* and *jun* proteins can be modified by a redox mechanism (22) in which a DNA repair enzyme reduces the sulphydryl group of a single cysteine residue to enhance DNA binding (23). Similarly NF-$\varkappa$B DNA binding activity, either in a purified form or in cell extracts was stimulated by the presence of reducing agents and abolished by exposure to sulphydryl modifying agents such as N-ethylmaleimide or iodoacetate (24, 25). Furthermore incubation of p50 with an oligonucleotide containing its specific DNA recognition sequence prior to exposure to the modification reagents protected the protein against inhibition of its DNA binding activity (24).

To directly demonstrate which amino acids were involved in the redox modulation of NF-$\varkappa$B DNA binding we have used a

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differential labelling protocol. [^{14}C] iodoacetate was used to chemically modify cysteine residues that had previously been protected from modification with unlabelled reagent by bound DNA. Sequencing of the [^{14}C] labelled tryptic peptides revealed that the bulk of the label had been incorporated into cysteine 62. The importance of this and other conserved cysteine residues in DNA recognition was determined by analysis of the DNA binding properties of purified p50 and mutated derivatives in which conserved cysteines had been changed to serine. While C119S and C273S mutants had relatively modest effects on DNA binding it was clear that the cysteine to serine change at residue 62 substantially reduced the affinity of this protein for its DNA recognition site. Competition experiments with a range of specific and non-specific DNA sequences indicated that the C62S mutant displayed an altered DNA binding specificity with a reduced ability to discriminate between specific and non-specific DNA.

MATERIALS AND METHODS

Expression of wild type and mutant p50 proteins in *E.coli*

As previously described (24), using a plasmid encoding the 105 kD precursor to p50 (kindly provided by A. Israël), a region of cDNA encoding amino acids 35 to 381 of p50 (roughly corresponding to the previously defined DNA binding and dimerisation region) was PCR amplified and ligated into the pGEX-2T expression vector (26) before transformation into *E.coli* JM101. For the construction of cDNAs encoding cysteine to serine mutations, a PCR mutagenesis technique was employed (27) with two internal mutagenising primers and the two original external primers to generate two partial length cDNAs, these in turn were used as the template for a second round of PCR using the external primers to generate full-length mutant cDNAs, these products were then ligated into pGEX-2T.

Protein purification

Essentially as described previously (24), *E.coli* JM101 transformants were grown in L-broth containing 50 μg/ml ampicillin to an A_{600} of 0.6, then GST-p50 fusion protein was induced with 0.5 mM IPTG for 4 h at 25°C. The cells were then pelleted, resuspended in ice cold 20 mM Na phosphate buffer pH 7.0, 0.5 M NaCl, 1 mM DTT (20 ml per litre of culture) and disrupted by sonication, all subsequent steps were at 4°C. Triton X-100 was added to 1%, the extract cleared by centrifugation, then applied to a glutathione-agarose column. After washing the column with extraction buffer, the fusion protein was eluted with buffer containing 10 mM reduced glutathione, 50 mM Tris−HCl pH 7.5, 0.5 M NaCl. The eluted fusion protein was cleaved by incubation with 6 U human thrombin per mg of fusion protein for 3 h at 20°C, then the NaCl concentration reduced to 0.25 M by dilution, and the mixture applied to a double-stranded calf thymus DNA−Sepharose column. After washing the column with 20 mM Na phosphate buffer pH 7.0, containing 0.2 M NaCl, 2 mM DTT, the bound protein was eluted by raising the NaCl concentration to 0.6 M. The peak fractions were aliquoted, frozen in liquid nitrogen, and stored at −70°C.

Differential labelling with [^{14}C] iodoacetate and identification of modified cysteine

Purified p50 protein was reduced by incubation with 20 mM DTT for 30 min on ice and diluted into 20 mM Na phosphate buffer pH 7.0 to reduce the NaCl concentration to 0.25 M before application to a recognition site DNA affinity column containing the NF-κB binding site present in the SV40 enhancer (6). The column was washed with 20 mM Na phosphate buffer pH 7.0, 0.25 M NaCl containing 1 mM DTT and bound protein was modified for 30 min at 20°C by equilibrating the column in the same buffer containing 5 mM iodoacetate. The reaction was quenched by initially washing the column with buffer containing 10 mM DTT and then re-equilibrating the column in buffer containing 1 mM DTT. Bound protein was eluted by raising the NaCl concentration to 0.6 M and the protein concentrated (Centricon-30 ultrafilter, Amicon) prior to labelling with 5 mM [^{14}C] iodoacetate (250 mCi per mmol, Amersham) for 30 min at 20°C. The reaction was quenched by the addition of DTT to 20 mM and dialysed overnight at 4°C against 20 mM Na phosphate pH 7.0 buffer containing 0.5 M NaCl, 1 mM DTT. [^{14}C]-labelled p50 subunit of NF-κB (400 pmol) was incubated with trypsin (0.4 μg) in 100 mM Tris−HCl pH 8.0 and 0.5 mM $CaCl_2$ in a total volume of 500 μl. After 8 h at 37°C another 0.4 μg of trypsin was added and the incubation continued for 16 h. The peptides were fractionated by reversed phase HPLC using a Brownlee™ C_{18} 220×2.1 mm ID column (Applied Biosystems). Solvent A was 0.1% aqueous TFA (Pierce) and solvent B was 0.1% TFA in acetonitrile (J.T. Baker). After sample injection (2×250 μl) the column was washed in solvent A for 2 min at a flow rate of 0.2 ml/min. A linear gradient of 0−40% solvent B was applied over 70 min. The column was washed with 60% solvent B for 5 min and re-equilibrated with solvent A. The elution of peptides was monitored by absorbance at 214 nm and peaks were collected manually. The radioactive peptides were identified by scintillation counting of 10% of the total volume and were dried without heat in a Speedvac. The pellets were resuspended in 25 μl 20% acetonitrile and 0.1% TFA and sequenced using an Applied Biosystems 477A pulsed-liquid phase instrument with an ABI-120 on-line PTH analyser. The TFA treated cartridge was coated with polybrene and precycled prior to sample application.

Gel electrophoresis DNA binding assay

Protein DNA-binding activity was determined using gel electrophoresis on 6% non-denaturing polyacrylamide (44:0.8 acrylamide:bisacrylamide) gels. Typically 1 μl of protein solution was fully reduced by addition of DTT to 25 mM incubated on ice for 15 min and added to 18 μl gel mobility shift binding buffer. This buffer contained 85 mM NaCl, 8.5% v/v glycerol, 22 mM Hepes-NaOH pH 7.5, 1.3 mg ml^{-1} BSA, 0.17% NP40, 3.6 mM spermidine (except when specifically omitted), 0.85 mM DTT, 0.85 mM EDTA, 6.1 mM $MgCl_2$) and the mixture incubated on ice for 15 min. Then ^{32}P-radiolabelled (T4 polynucleotide kinase, Amersham) double-stranded, blunt-ended 16-mer κB motif oligonucleotide (5′-CTGGGGACTTTCCAG-G-3′) was added and the mixture incubated for 15 min at 20°C before electrophoresis at 200 V for 40 min in 0.5×TBE buffer. After electrophoresis, gels were dried on DEAE-cellulose paper (Whatman, DE81), autoradiographed, and the amount of radioactivity in the DNA-protein complex gel slices determined by liquid scintillation counting (Ecoscint A, National Diagnostics). For determination of the dissociation constants for the various protein species in the presence of spermidine, a wide concentration range of binding site oligonucleotide was used with a constant protein concentration in an attempt to approach binding saturation for the protein. After incubation for 15 min at 20°C, the mixtures were electrophoresed on non-denaturing gels as

above, and the amount of radioactivity in the DNA-protein complex and free oligonucleotide bands determined by liquid scintillation counting. Values for dissociation constants were determined by Scatchard analysis. In the case of experiments determining the dissociation rate constants of the various p50 species in the presence or absence of spermidine, preformed DNA-protein complexes were challenged at 0°C with a 100-fold molar excess of unlabelled HIV-L oligonucleotide (see Table I for sequence), then after an appropriate interval samples were loaded onto a running non-denaturing gel.

RESULTS

Identification of cysteine residues involved in DNA binding

It has previously been demonstrated that incubation of purified p50 with iodoacetate abolished the DNA binding activity of the protein in a dose dependent fashion (24). However incubation of the p50 with a DNA fragment containing its specific recognition site prior to iodoacetate treatment abrogated its inhibitory effect (24). To identify amino acid residues involved in DNA binding we have employed a modification of the differential labelling procedure in which protein bound to its specific ligand is modified in the presence of iodoacetate such that the exposed residues are carboxymethylated. (Under the conditions employed in the following series of experiments iodoacetate predominantly modifies cysteines.) The ligand is then removed and the cysteine residues exposed are labelled with [^{14}C] iodoacetate. In this case the ligand is the specific DNA recognition sequence of the p50 subunit of the transcription factor NF-κB. Using this experimental protocol, 0.95 mol of [^{14}C] iodoacetate was incorporated per mol of p50, indicating that a single site in the protein was modified. p50 labelled using this procedure was subjected to trypsin digestion and the digest fractionated by reversed phase HPLC to reveal approximately

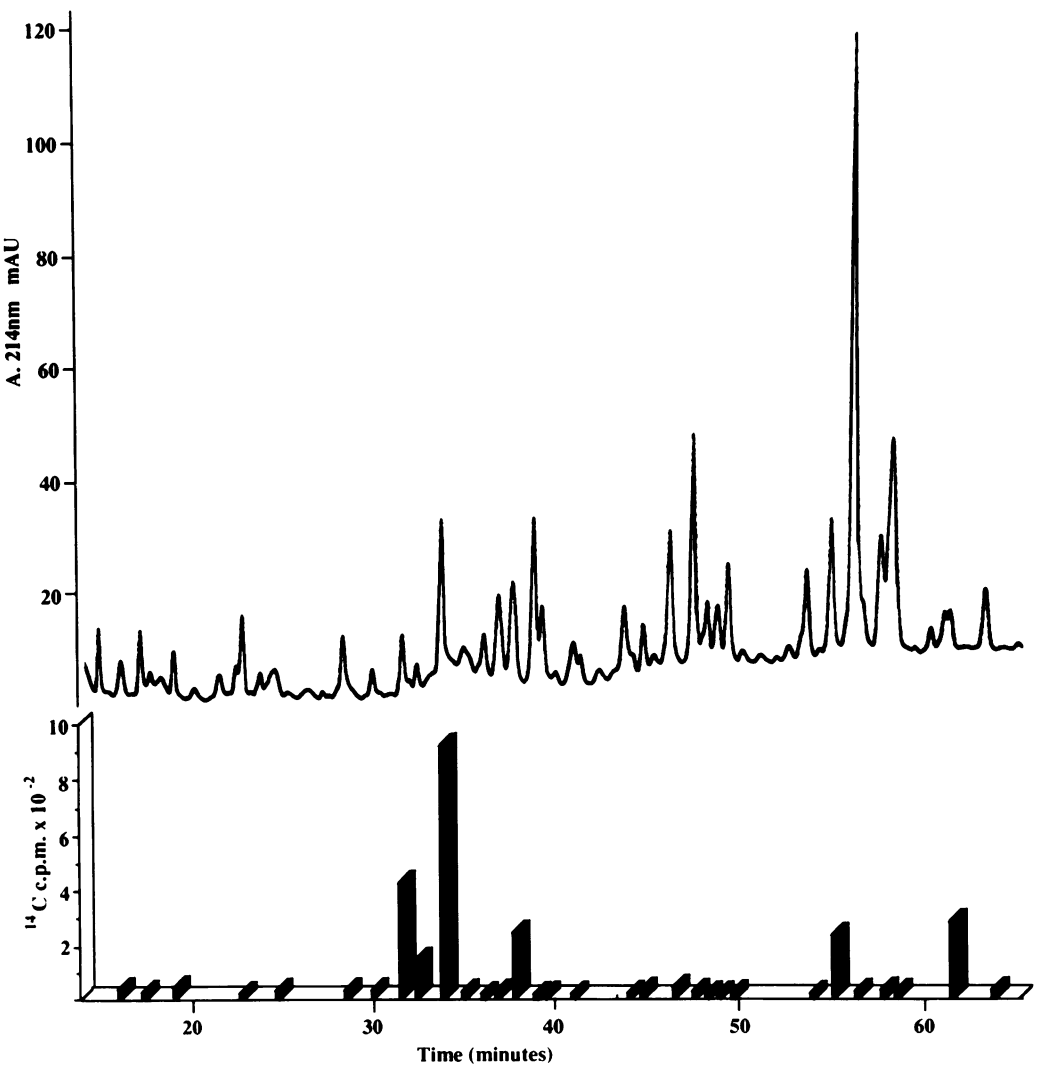

Figure 1. C_{18} reversed phase elution profile of trypsin digested, [^{14}C]-labelled p50. p50 bound to a DNA affinity column was treated with unlabelled iodoacetate. The column was washed to remove unreacted iodoacetate and bound protein eluted by raising the NaCl concentration to 0.6 M. Eluted protein was labelled with [^{14}C] iodoacetate, digested with trypsin and applied to a C_{18} column which was developed with a 0–40% gradient of acetonitrile in 0.1% TFA. Absorbance was monitored continuously at 214 nm and peaks were collected manually. The [^{14}C] radioactivity present in each peak was determined by liquid scintillation counting of 10% of each fraction. Peak numbers containing [^{14}C] radioactivity are indicated.

A

Peptide sequence	Peak number in tryptic digest	cpm in peptide	^{14}C-carboxy -methyl Cys
YVCEGPSHGG...	8	422	62
insufficient material	9	143	
YVCEGPS...	10	925	62
TAGCVTGGEEIY...	14	241	262
KSDLETSEPKPF...	14		-
GYNPGLLVHP...	26	232	-
TAGCVTGGEEIYLL	30	283	262
CDK...			

B

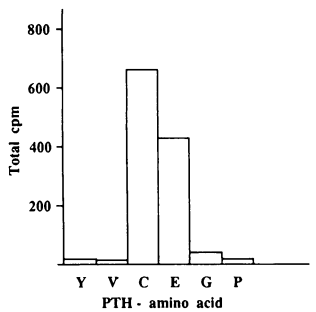

Figure 2. Sequence of [^{14}C]-labelled peptides of p50. **A.** [^{14}C]-labelled peptides, isolated by reversed phase fractionation on C_{18} (Figure 1), were dried and subjected to sequence analysis. Radioactivity in peptides was measured by scintillation counting of 10% of each A_{214} peak. **B.** Radioactivity in individual PTH-amino acid derivatives from the sequencing of tryptic peptide number 10.

thirty peaks of which six contained the [^{14}C] label (Figure 1). Of these six peaks the majority (66%) of radioactivity was found in peptides 8–10 which all contain Cys62 and are products of an incomplete digestion (Figure 2A). The phenylthiohydantoin (PTH) derivatives of the amino acids were collected during each Edman degradation cycle, and scintillation counted. In each case, the [^{14}C] label corresponds to the carboxymethyl Cys62-PTH (Figure 2B). Peptides 14 and 30 are also products of an incomplete digestion and in both cases the label is eluted as the carboxymethyl Cys262-PTH (Figure 2A), 23% of the total radioactivity was incorporated into this residue. Peptide 14 also co-eluted with a non-radioactive peptide which does not contain any cysteines. The residual [^{14}C] label was contained in peptide 26. It was not possible to determine with which amino acid the radioactivity was associated. His173 is a strong candidate since histidine is known to be modified by iodoacetate with a low efficiency, however no radioactivity was detected in the cycle in which the His173-PTH products eluted. In any case it seems clear that the majority of the incorporated [^{14}C] label corresponds to Cys62-PTH, with the conclusion that p50 Cys62 is intimately involved in contacting the specific DNA recognition sequence.

Determination of dissociation constants

To evaluate the contribution of individual cysteine residues to the affinity of p50 for DNA three mutants in which conserved cysteines were changed to serine were examined (Figure 3A, B). Dissociation constants were measured for the interaction of the 16bp wild type κB motif oligonucleotide (Table I) with wild type p50 (amino acid 35–381) and the C62S, C119S, and C273S mutant proteins (at 20°C and in the presence of spermidine). The K_D values obtained from analysis of Scatchard plots for the wild type and mutant proteins (Figure 3C) indicate that modification of cysteines at positions 119 and 273 had a relatively modest effect on DNA binding, whereas modification of cysteine 62 to serine resulted in a K_D value that was 10-fold higher than wild type.

Determination of dissociation rate constants

Wild type and mutant proteins were allowed to interact with ^{32}P labelled DNA and once equilibrium had been reached the dissociation rate constant was determined from the amount of ^{32}P labelled DNA remaining bound to the protein as a function of time after addition of a 100-fold molar excess of unlabelled DNA. As is the case with the K_Ds, the dissociation rate constants for wild type, C119S and C273S are similar, but the rate at which C62S dissociates from DNA in the presence of spermidine is so rapid that it cannot be measured (Figure 4C, D). Although the K_D values for the interaction between specific DNA sequences and the wild type and mutant p50 proteins in the presence or absence of spermidine are similar, the absolute values for the dissociation rate constants for the wild type, C119S, and C273S p50 proteins in the absence of spermidine were approximately 10-fold lower than in its presence (Figure 4D). Also, in the absence of spermidine it was now possible to determine an approximate value for the dissociation rate constant for the C62S mutant p50 protein of 0.35 s^{-1}. This suggests that the C62S mutant p50 protein also has a significantly higher association rate constant than the other protein species in the absence of spermidine.

Change in binding site specificity of the aa62 mutant

Given the changes in dissociation constant and kinetic behaviour of the C62S mutant p50 protein towards the κB motif, it seemed possible that the DNA binding site specificity of the C62S p50 mutant might have altered. Thus wild type and C62S mutant proteins were assayed for the ability of various oligonucleotide variants of the κB motif (Table I) to compete with the blunt-ended, double-stranded 16bp, wild type κB motif. Plots of relative amounts of radioactive DNA-protein complex remaining versus the dilution factor, defined as

$$1 - \left(\frac{M_l}{M_l + Mc} \right)$$

where M_l is the molarity of labelled oligo and M_c is the molarity of competitor oligo, should yield a straight line of gradient = −1 when the radiolabelled oligonucleotide probe is competed with identical unlabelled oligonucleotide. If the competitor oligonucleotide represents a poorer binding site for p50, the data points will fall above the straight line, whereas for a better competitor κB motif the data points will fall below the straight line. Competition specificity experiments were carried out in the presence of spermidine with a variety of oligonucleotide sequences and the relative affinities determined (Figure 5 and Table I). Competition of the radiolabelled, blunt-ended double-stranded 16bp wild type κB motif by the HIV-L oligonucleotide should yield a straight line plot but it is clear that the HIV-L oligo is a slightly poorer competitor (Figure 5, Table I). One significant difference between these two oligonucleotides is the number of base pairs the κB motif is from the end of the double-

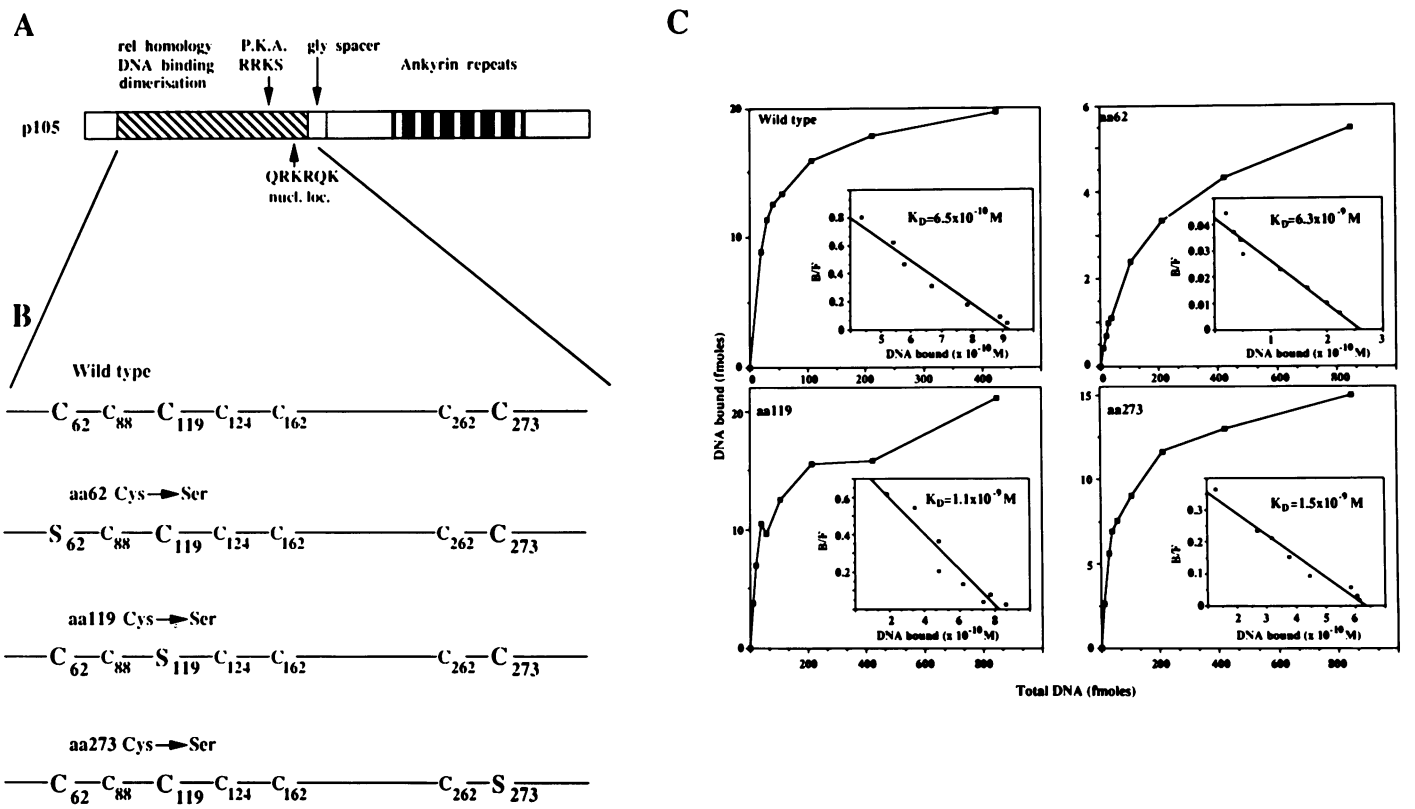

Figure 3. DNA binding properties of p50 cysteine to serine mutants. A. Diagram of the p105 precursor to the p50 NF-κB subunit showing the rel homologous region responsible for the DNA binding and dimerisation activity of p50 (hatched), a potential protein kinase A recognition sequence, a nuclear localisation signal known to be functional in rel proteins, the flexible glycine-rich spacer region, and the repeated motifs first identified in ankyrin and cell-cycle control proteins. **B.** Shows the amino acid 35 to 381 region of p105 which was expressed in *E.coli* JM101 and the position of the 7 cysteine residues within this region. Those cysteine residues completely conserved throughout all the members of the rel protein family are emphasised in bold, as are the positions of the various serine residues introduced into the protein by mutagenesis. C. Specific DNA binding properties of p50 and the three cysteine to serine mutants. Data obtained from gel electrophoresis DNA binding assays was used to construct binding curves and derive Scatchard plots for the various protein species as described in Materials and Methods.

Table I. Relative affinity of wild type and C62S mutant p50 for various κB related DNA sequences.

Oligo	Nucleotide Sequence	Rel.Molarity 50% Binding	
		W.T.p50	aa62 p50
16bp WT κB	5' CTG **GGGACTTTCC** AGG 3' 3' GAC **CCCTGAAAGG** TCC 5'	-	-
HIV-L	5' GATCTA **GGGACTTTCC** GCG 3' 3' AT **CCCTGAAAGG** CGCCTAG 5'	1.81	1.81
IRE	5' GATCAAAGT **GGGAAATTCC** TCTG 3' 3' TTTCA **CCCTTTAAGG** AGACCTAG 5'	1.35	1.35
H2TF1	5' GATCT **GGGGATTCCCC** AG 3' 3' A **CCCCTAAGGGG** TCCTAG 5'	0.40	1.96
EBP'cons'	5' GATCATG **GGGAATTTCCC** CAG 3' 3' TAC **CCCTTAAAGGG** GTCCTAG 5'	0.30	0.58
SVUP	5' GATCTGAGGC **GGAAAGAACC** AGCTG 3' 3' ACTCCG **CCTTTCTTGG** TCGACCTAG 5'	39.0	9.64
SV1-M1	5' GATCTAGGGTGT **CCAAAGTCCC** G 3' 3' ATCCCACA **GGTTTCAGGG** CCTAG 5'	54.5	6.25
SV1-M2	5' GATCTAGGGTGT **GGAATGTCCC** G 3' 3' ATCCCACA **CCTTACAGGG** CCTAG 5'	25.3	4.00
SV1-M3	5' GATCTAGGGTGT **GGAAAGTGGC** CG 3' 3' ATCCCACA **CCTTTCACCG** GCCTAG 5'	199	27.6

Shown are the sequences of the double-stranded oligonucleotides used in this study, and the relative molarities of competitor oligonucleotides needed to reduce the amount of radiolabelled wild type and C62S p50–16-mer κB oligonucleotide complex by 50%.

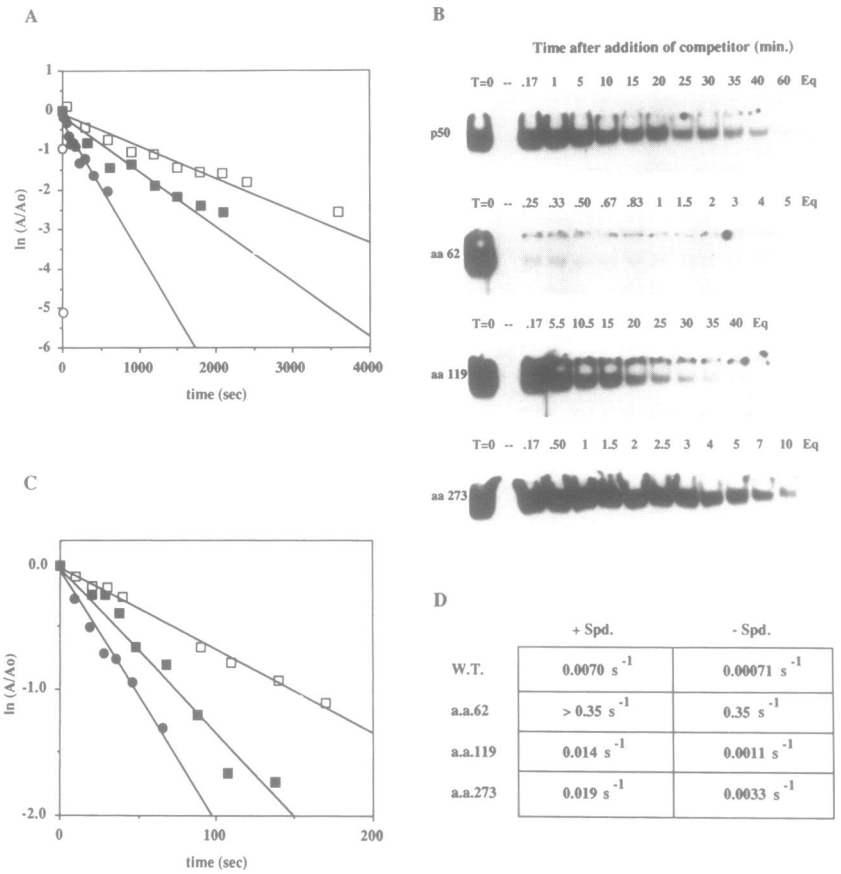

Figure 4. Determination of the dissociation rate constants for p50 and the three cysteine to serine mutants in the presence and absence of spermidine. Complexes of p50 proteins formed with [^{32}P] labelled 16-mer κB oligonucleotide at 0°C either in the absence (**A**, **B**) or presence (**C**) of 3.6mM spermidine were mixed with a 100-fold molar excess of unlabelled HIV-L oligonucleotide and after the indicated times the amount of [^{32}P] labelled DNA remaining bound to protein was determined by gel electrophoresis. The data was plotted as the integrated pseudo first order rate equation, ln (A/Ao) versus time where Ao represents the amount of DNA-protein complex present at the first timepoint after addition of unlabelled DNA and A represents the amount of DNA-protein complex remaining after the indicated time. The symbols used were: open square wild type p50; open circle C62S; solid square C119S; solid circle C273S. 'Eq' represents the amount of radiolabelled DNA-protein complex which would be seen at equilibrium at infinite time. **D.** Dissociation rate constants for p50 protein−16-mer κB motif oligonucleotide complexes at 0°C in the presence and absence of 3.6 mM spermidine derived from the gradients of the integrated pseudo first-order rate equation ln (A/Ao) versus time.

stranded region: three and two base pairs respectively. While wild type and C62S show similar specificity towards the HIV-L and IRE DNA sequences, wild type p50 displays a higher affinity for the H2TF1 sequence than the HIV-L sequence and the reverse is true for C62S (Figure 5, Table I). In the case of the EBP cons type of κB motif, (Table I), this is a significantly better competitor for the wild type protein, with the C62S mutant protein having a relatively poorer affinity for this sequence—although still higher than its affinity for the wild type κB 16bp oligonucleotide. Mutant κB sites SVUP, SV1-M1, SV1-M2 and SV1-M3 are poor competitors for binding of both the wild type and C62S mutant p50 proteins but show higher binding affinities towards the C62S mutant p50 protein. On the basis of these competition studies, the C62S mutant p50 protein clearly has an altered binding site specificity.

Ability of wild type and mutant p50 proteins to discriminate between specific and non-specific DNA

Wild type p50, and the C62S, C119S, and C273S mutants were incubated with ^{32}P labelled DNA (HIV-L) containing the specific DNA recognition site in the presence of a range of concentrations of unlabelled non-specific DNA (sheared double stranded *E.coli* DNA) and the amount of ^{32}P labelled DNA remaining in the DNA-protein complex determined by native polyacrylamide gel electrophoresis. Analysis of the data in Figure 6 indicates that while the wild type, C119S and C273S proteins all have similar abilities to discriminate between specific and non-specific DNA the C62S mutant is impaired in this respect: the amount of *E.coli* DNA required to reduce specific DNA binding by 50% is fivefold less for the C62S mutant than for either the wild type, C119S or C273S mutants. Although the DNA binding specificity assays (Figures 5, 6 and Table I) were all carried out in the presence of spermidine no significant differences in DNA binding specificity were observed when these assays were carried out in the absence of spermidine (data not shown).

DISCUSSION

The differential labelling experiment described here represents a powerful approach to the identification of amino acid residues involved in DNA-protein interactions. Although the procedure described here is relatively specific for cysteine residues the availability of a large number of well characterised group specific

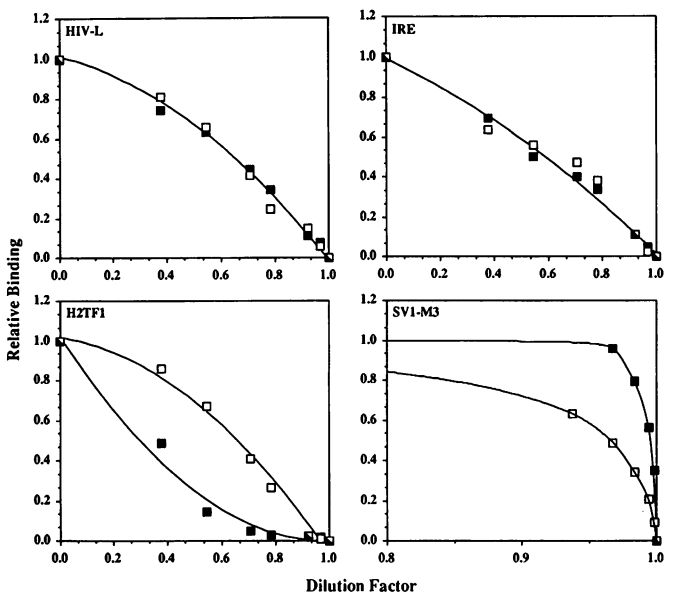

Figure 5. DNA binding specificity of wild type and C62S p50 proteins. Competition analysis was used to determine the relative affinity of the wild type and mutant protein for the κB related DNA sequences in the HIV-L, IRE, H2TF1 and SV1-M1 oligonucleotides. A summary of the data and the DNA sequences used is shown in Table I. Radiolabelled 16-mer κB oligonucleotide was mixed with the indicated unlabelled oligonucleotides and wild type or C62S p50 protein added. Once equilibrium had been reached free DNA was separated from DNA-protein complexes on a native polyacrylamide gel and the radioactivity in the DNA-protein complex determined. Plots of relative amounts of radioactivity in the DNA-protein complex remaining versus the dilution factor (defined as

$$1-\left(\frac{M_l}{M_l + Mc}\right)$$

where M_l is the molarity of the labelled DNA and M_c is the molarity of the competitor DNA. Filled squares represent wild type p50 and open squares represent C62S.

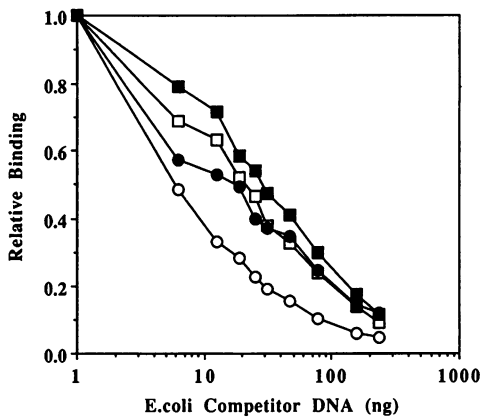

Figure 6. Competition of the specific binding of all four p50 protein species by non-specific, sheared, *E.coli* chromosomal DNA. The specific κB probe used in this experiment was Klenow fragment labelled HIV-L oligonucleotide. The amount of ^{32}P labelled oligonucleotide remaining in the DNA-protein complex was determined in a native polyacrylamide gel as described in the legend to Figure 5 and is expressed relative to the amount of radiolabelled DNA-protein complex in the absence of *E.coli* competitor DNA. Symbols used for the various p50 protein species in the plots were: wild type open square; C62S open circle; C119S solid square; C273S solid circle.

reagents will allow this approach to be extended to other amino acids that interact with DNA. Chemical modification with [^{14}C]-labelled iodoacetate indicated that cysteine 62 in the p50 subunit of NF-κB was protected by bound DNA from initial modification by unlabelled iodoacetate and implied that this amino acid residue is in close proximity to the bound DNA. This prediction was confirmed by site-directed mutagenesis in which the mutation of cysteine 62 to serine resulted in a protein with reduced affinity for its specific DNA recognition site and which was also impaired in its ability to discriminate between specific and non-specific DNA. Thus it seems clear that carboxymethylation of cysteine 62 is responsible for the loss of DNA binding activity observed when p50 is exposed to iodoacetate (24). It is possible however that the partial protection noted on cysteine 262 and histidine 173 could indicate their involvement in DNA binding, but it is equally possible that these residues were rendered partially inaccessible to iodoacetate as a result of a conformational change associated with DNA binding. The tenfold higher dissociation constant measured for specific DNA binding of the C62S p50 mutant represents a weakening of binding energy by 1.32 kcal mol^{-1} at 20°C and is consistent with the loss of a single neutral hydrogen bond (28). While the cysteine to serine alteration is conservative, merely involving the substitution of sulphur with oxygen it may be that the change in local geometry accompanying this amino acid substitution is responsible for the inability of the serine to act as a hydrogen bond donor—for example the optimum O-H···O hydrogen bond distance is at least 0.04 nm shorter than the corresponding S-H···O distance (29). The dissociation constant values obtained here for the interaction of p50 with specific DNA are of the same general order as those observed for many other sequence-specific DNA-binding proteins. However, the dissociation constants are somewhat higher than those obtained by other workers with native NF-κB proteins: 2.7×10^{-12} M for affinity purified NF-κB (30), 4×10^{-13} M for renatured, gel-purified p50-p65 complex and 9×10^{-13} M for the renatured, gel-purified p50 homodimer (31). It is possible that native proteins purified from eukaryotic cells carry post-translational modifications that influence their DNA binding behaviour, alternatively the differences may be due to the use of a partial length p50 construct. Quantitative DNA binding studies with recombinant NF-κB proteins and a variety of specific DNA recognition sites have reported (20, 21, 32) a wide range of values for dissociation constants (6.7×10^{-12} to 2.9×10^{-10} M). Although the value obtained is dependent on the precise DNA recognition sequence, the nature of the NF-κB recombinant protein and the conditions under which the assays were performed it is clear that the values described here fall within this broad range.

In both the presence and absence of spermidine, the differences in the dissociation constant values for the wild type, C119S, and C273S mutant p50 proteins seem to be explicable in terms of altered dissociation rate constants, and hence the prediction that they have similar association rate constants. Thus, in the presence of spermidine, the association rate constants for the wild type, C119S, and C273S mutant proteins would be predicted to be in the region of 1×10^{7} $M^{-1}s^{-1}$ at 0°C if the overall dissociation constants remain broadly unchanged between 0 and 20°C. In contrast, the C62S mutant protein in the presence of spermidine

has a disproportionately larger dissociation rate constant and a larger association rate constant compared to the three other species—one possibility is that this may reflect a different mechanism of complex formation. In the absence of spermidine, the differences in the predicted values for the dissociation constants for the wild type, C119S, and C273S proteins would seem to be explicable by the differences in dissociation rate constants. Given the approximately 10-fold lower values for the wild type, C119S, and C273S proteins' dissociation rate constants in the absence of spermidine, the association rate constants for these proteins in the absence of spermidine ought to be in the order of $1-2\times10^6$ $M^{-1}s^{-1}$ at 0°C. Again, the C62S mutant protein in the absence of spermidine shows a disproportionately large dissociation rate constant (approximately 500-fold larger than the 0.00071 s^{-1} value for the wild type p50), and would be predicted to have a larger association rate constant (of approximately 2×10^7 $M^{-1}s^{-1}$ at 0°C) compared to the wild type, C119S, and C273S proteins. This increase in association rate for the C62S mutant could be due to a specific interaction between the hydroxyl and nucleic acid in the encounter transition state, or simply enhancement of the rate of isomerisation of the complex to produce a tightly bound product.

Observations of increased dissociation rate constants and predicted increases in association rate constants in the presence of spermidine are not unique to the p50 system as it was previously noted that for baculovirus expressed serum response factor the rate of binding to, and dissociation from, the c-*fos* serum response element was increased by the presence of 3 mM spermidine (33).

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