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DNA binding alters the protease susceptibility of the p50 subunit of NF-κB

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ABSTRACT

The subdomain structure of the p50 subunit of NF-κB (amino acids 35–381) was investigated by partial proteolysis of the native protein. Trypsin cleaves p50 at a limited number of sites with an initial cleavage at low trypsin concentration occurring after R362 and a second cleavage taking place at higher trypsin concentration after K77. The cleavage after R362 does not alter the DNA binding characteristics of p50 but removes the nuclear localisation signal indicating that this region occupies a highly exposed position on the surface of the protein. The second cleavage after K77 generates a protein that although dimeric is incapable of binding DNA, thus emphasising the importance of residues 35–77 in DNA recognition. However p50 dimers containing one molecule cleaved after K77 and one molecule with this region intact are capable of binding DNA. When very high concentrations of trypsin are employed p50 is completely degraded. However if p50 is bound tightly to DNA containing its specific recognition site prior to trypsin addition the cleavage after K77 is almost completely blocked and the protein becomes highly resistant to proteolysis. These data suggest that bound DNA may mask critical trypsin cleavage sites or that DNA binding is accompanied by a conformational change in protein structure that renders the protein resistant to proteolysis.

INTRODUCTION

The NF-κB family of transcription factors participates in the inducible expression of a wide variety of viral and cellular genes. In uninduced cells NF-κB is sequestered in an inactive state but is rapidly activated after exposure to a wide variety of stimuli. Transcription directed by the LTR of the human immunodeficiency virus type 1 (HIV1), for example, takes place a low level in uninduced T-cells but is strongly induced by cytokines or T-cell activation signals which activate NF-κB (1). Prototypical NF-κB is a dimer of two polypeptides of 50 000 (p50) and 65 000 (p65) molecular weight that binds to ten base pair recognition sites with each subunit contributing to the specificity of the heterodimer (2). p50 can also exist in a homodimeric form that is constitutively active in the nuclei of a variety of cell types(3–5). Both p50 and p65 contain a large N-terminal domain, responsible for the DNA binding and dimerisation activities of the proteins, that is homologous to the c-*rel* protooncogene and the *Drosophila* maternal effect gene dorsal (6–12). Additional members of this family of transcription factors that have recently been identified which contain the *rel* homologous region include RelB (13) and NFKB2 (also known as lyt-10, p50B, p49, 14–16). While both p50 and p65 subunits contribute to DNA binding it appears that in vivo it is the p65 component of the heterodimer that is responsible for the transcriptional activity of NF-κB (17–20) although addition of purified p50 homodimers to an in vitro reaction resulted in a strong activation of transcription (21–22).

Although the DNA binding form of NF-κB is found in the nucleus of activated cells it is not present in the nucleus of uninduced cells. Activation of NF-κB can take place in the absence of protein synthesis (23) and treatment of cytoplasmic extracts from uninduced cells with agents capable of dissociating protein-protein interactions results in the appearance of DNA binding activity (24). Once dissociated the inhibitor of NF-κB, known as IκB, was isolated and shown to reversibly inhibit the DNA binding activity of heterodimers of p50 and p65 but not homodimers of p50 (25–28). Like the DNA binding subunits the IκB's constitute a family of proteins that have distinct specificities for the various homo and heterodimers of *rel* related proteins. At least three distinct cDNAs coding for MAD3, bcl-3 and IκBγ have been shown to possess IκB activity and all possess a series of related sequences known as ankyrin repeats that are also found in cell cycle control proteins (29–34). Although the mechanisms have yet to be determined these proteins appear to act by binding to the *rel* homologous regions of the NF-κB proteins and simultaneously altering their nuclear, cytoplasmic distribution and DNA binding activities (35, 36).

A further level at which the activity of NF-κB can be controlled was revealed when the genes encoding p50 were isolated (7, 8, 14, 37). In fact the gene for p50 encodes a protein of 105 000 molecular weight (p105) and p50 is released from this protein by proteolytic processing (38). The C-terminal half of p105 also contains ankyrin repeats and IκBγ is produced from this same gene by alternative splicing (33, 34). p105 therefore contains an IκB activity that can act in *cis* and for this reason the intact protein is found in the cytoplasm, associated with other rel related proteins, in a form that is unable to bind DNA (32, 39, 40). Each of the NF-κB proteins contain a functional nuclear localisation

signal at the C-terminal end of the *rel* related region that appears to be masked when IκB molecules are bound. While this interaction can retain the proteins in the cytoplasm by simply blocking the protein's ability to interact with the nuclear transport apparatus it is less clear how the DNA binding inhibition is accomplished as the nuclear localisation signal is dispensable for DNA binding (35). It is therefore clear that the *rel* homologous region is responsible for a number of distinct functions including nuclear localisation, DNA binding, dimerisation and IκB binding. The objective of this work was therefore to investigate the subdomain structure of the p50 subunit of NF-κB in an attempt to ascribe the various functions to distinct regions of the protein. In an approach complementary to genetic analysis we have probed the structure of purified and active p50 (amino acids 35−381) using partial proteolysis. Under controlled conditions trypsin cleaves initially after R362 then after K77. The cleavage after R362 does not alter the DNA binding characteristics of the protein but removes the nuclear localisation signal and indicates that this region of the protein is in a highly accessible environment on the surface of the protein. In contrast cleavage after K77, which removes the N-terminal 43 amino acids, abolishes DNA binding but if the protein is first bound to DNA this cleavage site is inaccessible. Furthermore at very high concentrations of trypsin p50 is completely degraded but becomes resistant to proteolysis if bound to DNA containing its specific recognition site. These data suggest that DNA binding may be accompanied by a conformational change in the structure of the protein that renders the protein resistant to proteolysis or alternatively that bound DNA masks critical trypsin cleavage sites.

MATERIALS AND METHODS

Expression and purification of NF-κB p50

Amino acids 35−381 of p50 were expressed in *E.coli* as part of a fusion protein with glutathione-S-transferase and purified by chromatography on glutathione agarose, thrombin cleavage and affinity chromatography on DNA-Sepharose as described previously (41).

Analytical procedures

Gel electrophoresis DNA binding assays, DNA affinity chromatography and SDS polyacrylamide gel electrophoresis were as described previously (41, 42).

Gel filtration

p50 and trypsin cleaved derivatives were injected onto a 9.4×250 mm Zorbax G250 hydrophilic gel filtration column installed in a Waters 600E HPLC system. The column was equilibrated in buffer containing 20 mM sodium phosphate pH 7.0, 0.5 M NaCl, 1 mM DTT and developed with the same buffer at a flow rate of 1 ml per minute. Absorbance at 280 nm was monitored continuously on a Waters 484 tuneable detector and peaks were collected manually. The void volume of the column was determined with Blue Dextran and the column calibrated as described (43) using the retention coefficient (V_e/V_o) and molecular weights of the following proteins: thyroglobulin, 669 000; apoferritin, 443 000; β-amylase, 200 000; bovine serum albumin, 66 000; ovalbumin, 48 000; cytochrome c, 12 4000.

Partial proteolysis

Purified p50 (10 μg, 3 μl) in 20 mM sodium phosphate pH 7.0, 0.5 M NaCl, 1 mM DTT was mixed with 1 μl of a dilution of trypsin (TPCK-treated, Sigma) in the same buffer and incubated at 20°C for 120 minutes. Digestion was terminated by the addition of 4 μl 2 mM freshly diluted PMSF and a further 15 minutes incubation at 20°C. Samples were denatured at 100°C for 2 minutes after addition of SDS and mercaptoethanol and analysed by SDS polyacrylamide gel electrophoresis. For N-terminal sequence analysis the proteins were electrophoretically transferred onto PVDF membrane (ProBlot, Applied Biosystems), visualised by briefly staining with Coomassie Brilliant Blue and the excised bands sequenced by David Campbell, University of Dundee. Samples for mass analysis were digested with trypsin to yield predominantly form I or form II molecules, the digestion terminated by t he addition of PMSF and the products collected by precipitation with 80% acetone. Mass analysis was conducted by Ann Dell, Imperial College of Science and Technology.

Partial proteolysis in the presence of DNA was conducted in 20 mM sodium phosphate pH 7.0, 0.15 M NaCl, 1 mM DTT using equimolar amounts of double stranded synthetic oligonucleotides and p50 (monomer). Digestion products were analysed as described above.

Chemical cross-linking

p50 form I and form II were prepared by trypsin digestion at 1:800 and 1:50 (w/w) ratios and the digestion terminated with 1 mM PMSF. Prior to diamide cross-linking all samples were reduced by incubation in the presence of 20 mM DTT and the buffer exchanged by passage through a spin column of Sephadex G50 equilibrated in 20 mM sodium phosphate pH 7.0, 0.5 M NaCl. p50 (10 μM) was crosslinked by the addition of diamide (44) to a final concentration of 100 μM and the reaction allowed to proceed for 15 minutes at 20°C. Samples were heated to 100°C in the presence of SDS, but in the absence of reducing agent. Products of crosslinking were separated in an 8% polyacrylamide gel and visualised by staining with Coomassie Brilliant Blue.

p50 (10 μM) in 0.5 M sodium borate pH 8.9 was cross-linked by the addition of glutaraldehyde to 1 or 2 mM and the reaction allowed to proceed for 20 seconds at 20°C before addition of sodium borohydride to 10 mM. Samples were heated to 100°C in the presence of SDS and mercaptoethanol then analysed by Coomassie Brilliant Blue staining after electrophoresis in 10% SDS polyacrylamide gels.

RESULTS

Limited cleavage of p50 by trypsin

In vivo the p105 precursor is proteolytically processed to generate an N-terminal DNA binding domain that represents the p50 subunit of NF-κB. p50 can exist in a homodimeric form or as a heterodimer complexed to p65 protein which also contains a *rel* homologous DNA binding domain. The DNA binding domain (amino acids 35−381, reference 8) of the p50 subunit of NF-κB was expressed in bacteria as a thrombin cleavable fusion with glutathione-S-transferase and purified to homogeneity as described previously (41). The purified protein contains the expected N and C termini (determined by N-terminal sequencing and mass analysis), is dimeric in solution and is fully active in DNA binding (42). To investigate the subdomain structure of p50 the purified protein was exposed to a range of concentrations of trypsin and the digestion products analysed by SDS polyacrylamide gel electrophoresis. It is immediately apparent that trypsin cleaves p50 at a limited number of sites with an initial

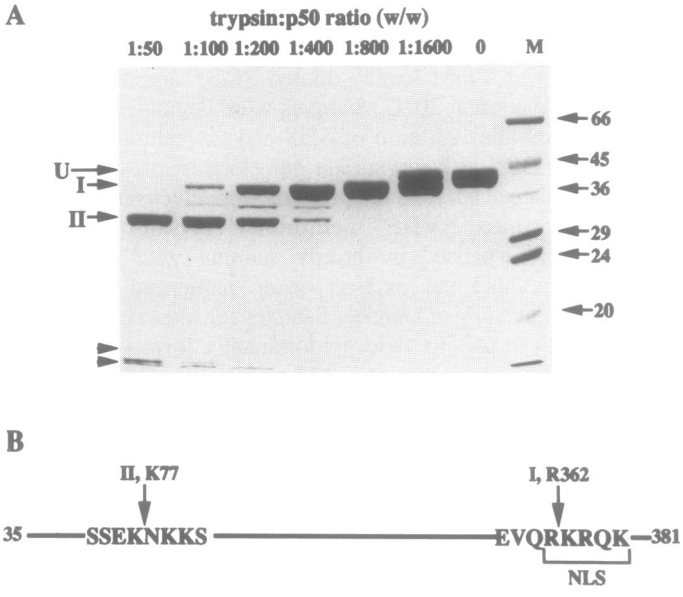

Figure 1. Limited trypsin cleavage of NF-$\varkappa$B p50. **A**, Trypsin was added to p50 (10 μg) at the ratio indicated in the figure and incubated at 20°C for 120 minutes after which time the reaction was terminated by the addition of PMSF. Digestion products and marker proteins (M) were fractionated in a 10% polyacrylamide gel containing SDS and visualised by staining with Coomassie Brilliant Blue. The positions at which untreated p50 (U), form I (I) and form II (II) migrate are indicated. **B**, Sites of trypsin cleavage in p50 determined by N-terminal sequencing and mass analysis.

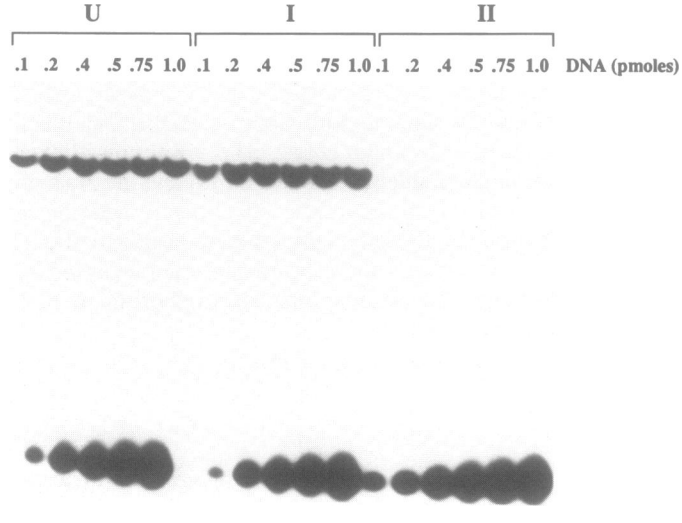

Figure 2. Sequence specific DNA binding of trypsin cleaved p50. 0.5 pmole (monomer) of untreated (U) or trypsin treated (I, II) p50 was incubated with the indicated amount of ^{32}P labelled DNA which contains the $\varkappa$B binding site present in the HIV LTR and free DNA separated from DNA-protein complexes by electrophoresis in a native polyacrylamide gel.

cleavage occurring at low trypsin concentration to generate a product designated as form I and a second cleavage taking place at higher trypsin concentration to generate a product designated as form II. An additional minor cleavage product is also observed at intermediate concentrations of trypsin (figure 1A). To map the sites at which these trypsin cleavages take place the form I and II molecules were subjected to N-terminal sequencing and

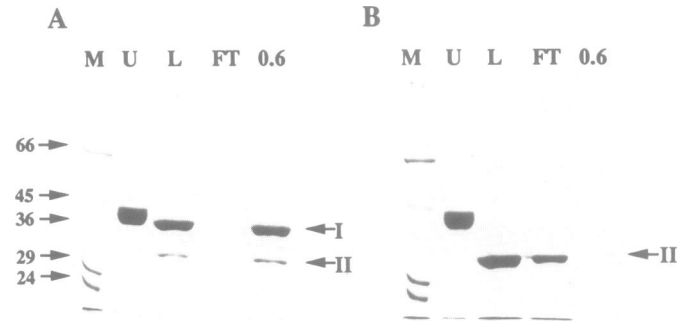

Figure 3. Non-sequence specific DNA binding of trypsin cleaved p50. p50 treated with either a 1:400 (**A**) or 1:50 (**B**) ratio of trypsin was applied to a column of double stranded calf thymus DNA Sepharose (L). The column was washed with buffer containing 0.25 M NaCl to remove protein which failed to bind (FT) and bound proteins eluted by raising the NaCl concentration to 0.6 M (0.6). Eluted fractions along with untreated p50 (U) and marker proteins were analysed in a 10% polyacrylamide gel containing SDS followed by staining with Coomassie Brilliant Blue.

mass analysis. The N-terminus of the form I molecule was GSNMALPTAD where the first three residues are derived from the cloning vector and represent the expected N-terminus of the protein generated after thrombin cleavage. N-terminal sequencing of the form II molecule generated the sequence NKKSYPQVKI that represents cleavage after K77. Mass analysis indicated that the single cleavage which generates the form I molecule takes place at the C-terminus of p50 within the nuclear localisation signal after R362. A second cleavage which occurs after K77 generates the form II molecule (figure 1B). Thus the nuclear localisation signal of p50 is located in a highly exposed position which renders it extremely sensitive to proteolytic digestion with trypsin.

DNA binding properties of trypsin cleaved p50

To determined the DNA binding properties of p50 that had been cleaved at a single site (form I) or at two sites (form II) samples which had been trypsin digested at 1:800 or a 1:50 trypsin to p50 ratio (figure 1) were prepared. The affinity of untreated and the two trypsin digested forms for the specific DNA recognition site ($\varkappa$B motif) present on a double stranded synthetic oligonucleotide was determined in a gel electrophoresis DNA binding assay. A defined amount of untreated and trypsin digested forms of p50 were incubated with increasing amounts of ^{32}P labelled DNA containing the specific recognition site and the fraction of labelled DNA incorporated into DNA-protein complex determined after separation from free DNA in a native polyacrylamide gel. While untreated and trypsin cleaved form I p50 have an indistinguishable affinity for DNA, form II p50 has lost all detectable DNA binding capacity (figure 2) indicating that the region between amino acids 35 and 77 is crucial for sequence specific DNA binding.

To determine if this same region was required for non-specific DNA binding p50 treated with trypsin to contain predominantly either form I or form II molecules was analysed by affinity chromatography on columns of double stranded calf thymus DNA Sepharose. Trypsin digested p50 was loaded onto the columns which were washed with buffer containing 0.25M NaCl to remove unbound proteins and bound proteins eluted by raising the NaCl concentration in the buffer to 0.6M. Fractions of the

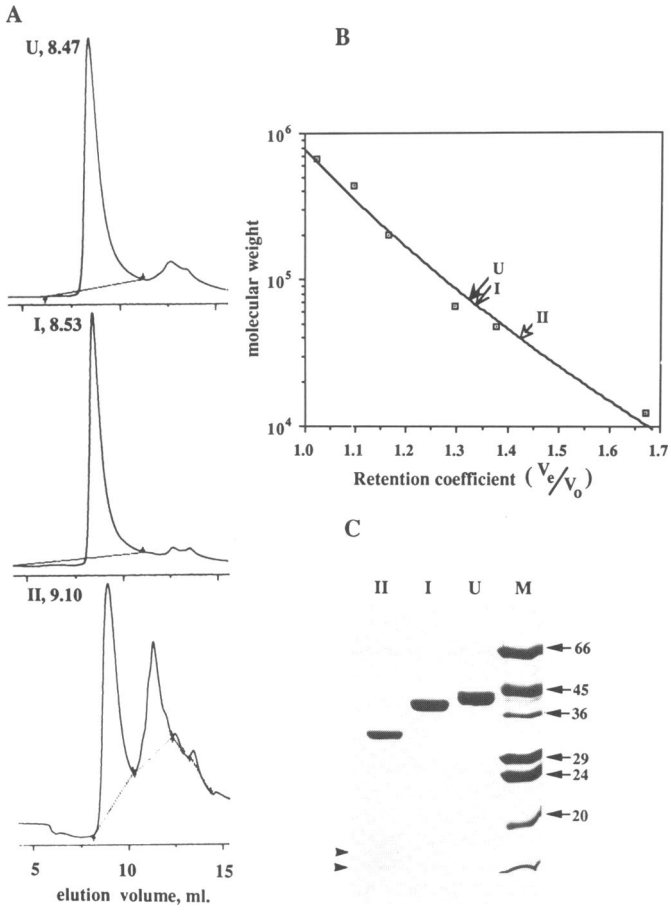

Figure 4. Gel filtration of p50 digestion products. Untreated p50 (U) or p50 treated with a 1:800 (I) or 1:50 (II) ratio of trypsin were applied to a Zorbax GF250 gel filtration column eluted at 1 ml per minute and the column eluate continuously monitored at 280 nm (**A**). From the indicated elution volumes retention coefficients were used to calculate apparent molecular weight (**B**). The column was calibrated by plotting the retention coefficient (V_e/V_o) against the $\log_{10}$ of the molecular weight of a set of standards of known molecular weight (Blue Dextran, thyroglobulin, apoferritin, β-amylase, bovine serum albumin, ovalbumin and cytochrome c). Peaks were collected manually and proteins present analysed by SDS polyacrylamide gel electrophoresis and staining with Coomassie Brilliant Blue (**C**).

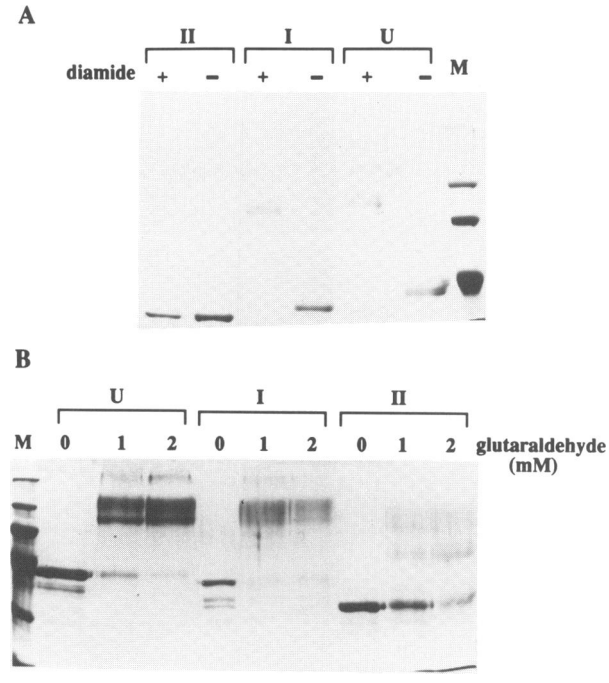

Figure 5. Chemical cross-linking of p50 digestion products. Untreated p50 (U) or p50 treated with a 1:800 (I) or 1:50 (II) ratio of trypsin were subjected to chemical cross-linking with diamide (**A**) or glutaraldehyde (**B**). Products of diamide cross-linking were fractionated in an SDS polyacrylamide gel under non-reducing conditions (A) while products of glutaraldehyde crosslinking were fractionated under reducing conditions (B). Proteins were visualised by staining with Coomassie Brilliant Blue. Molecular weight markers (M) were 200, 97, 68, 43 and 29 kD.

load (L), flow through (FT) and 0.6M NaCl eluate (0.6) were analysed by SDS polyacrylamide gel electrophoresis (figure 3). Form I material binds efficiently to the DNA and is not found in the flow through fraction but can be eluted with 0.6M NaCl (figure 3A). In contrast form II material does not bind to the DNA column and as a consequence is found in the flow through fraction but not the 0.6M NaCl elution (figure 3B). Thus the same region between residues 35 and 77 that is required for sequence specific DNA binding is also required for non-specific DNA binding. However it should be noted that when a mixture of form I and II material is applied to the DNA column the form II material now binds to the column and elutes with 0.6M NaCl (figure 3A). As p50 exists as a homodimer in solution (see below) it is likely that when the p50 dimer contains two form II molecules it is incapable of binding DNA whereas a p50 dimer containing one molecule of form I and one of form II it is still capable of binding to DNA.

Dimerisation of trypsin treated p50

As p50 is dimeric in solution and when bound to DNA the multimeric state of untreated and trypsin cleaved p50 was determined by gel filtration and chemical cross-linking. Each of the three forms of p50 was analysed by HPLC gel filtration in a buffer containing 0.65 M NaCl and the apparent molecular weight determined from the retention coefficient. Both untreated and form I elute from the column with retention coefficients that correspond to molecular weights of 77,000 and 72,000 respectively (figure 4A, B), close to the predicted dimer molecular weights of 77,818 and 73,888. However the form II material elutes from the column later with an apparent molecular weight of 40,000 (figure 4A, B). Although this apparent molecular weight is larger than the predicted monomer molecular weight of 32,017 it is substantially less than would be expected if the form II material remained as a dimer. Analysis of the peak fractions by SDS polyacrylamide gel electrophoresis reveals the expected products but it should be noted that two of the small fragments generated by the action of trypsin coelute with the form two material (figure 4C). Thus, like the untreated material, form I protein is clearly dimeric in solution although the situation is less clear in the case of the form II material.

To resolve this issue chemical cross-linking with diamide, which promotes the formation of disulphide bonds between adjacent cysteine residues or glutaraldehyde which cross-links protein amino groups was employed. Untreated p50, form I and form II were treated with diamide, the reaction terminated with iodoacetate and the products analysed by SDS polyacrylamide

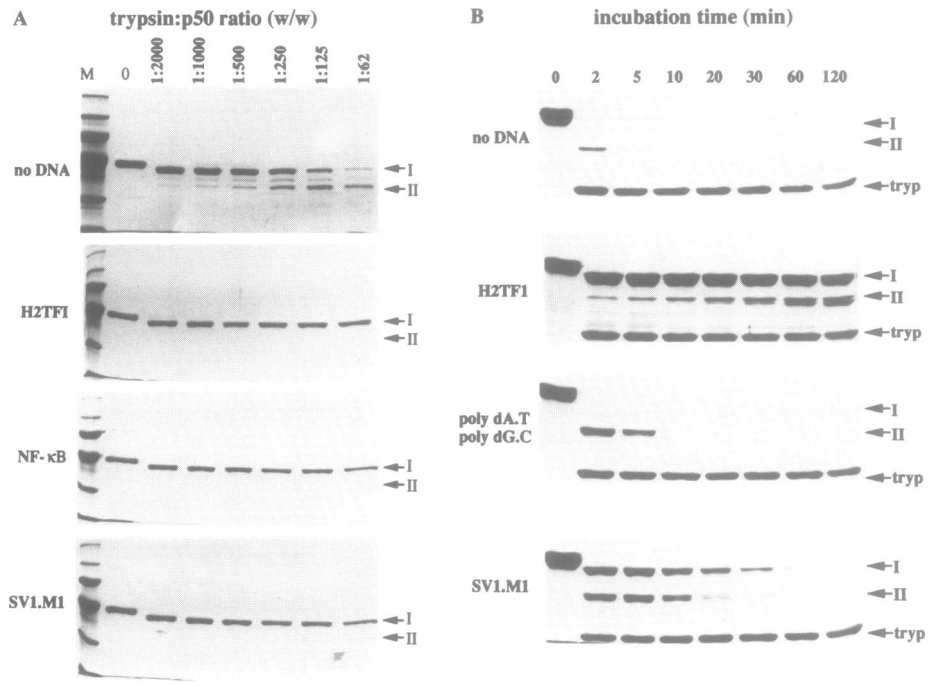

Figure 6. Effect of bound DNA on trypsin cleavage of p50. **A.** Prior to digestion (20°C, 120 minutes) with the indicated amounts of trypsin p50 (150 pmole, monomer) was incubated in the absence of DNA or with a double stranded synthetic oligonucleotide (150 pmole) containing a symmetrical κB motif (H2TF1), a non-symmetrical κB motif (NF-kB) or a mutated κB motif (SV1.M1). **B.** p50 (300 pmole) was incubated either in the absence of DNA or in the presence of non-specific DNA (poly dA.T, poly dG.C, 7.5 nmole, nucleotide), high affinity specific DNA (H2TF1, 300 pmole) or low affinity specific DNA (SV1.M1, 300 pmole). Trypsin at a 1:3 ratio (w/w) was added and the digestion allowed to proceed at 20°C for the times indicated. Digestion products were analysed in 10% polyacrylamide gels followed by staining with Coomassie Brilliant Blue. The positions of p50 form I (I), p50 form II (II) and trypsin (tryp) are indicated. Marker proteins are as described in the legend to figure 5.

gel electrophoresis under non-reducing conditions. While the untreated and form I species efficiently form disulphide linked dimers, treatment of form II molecules does not generate any dimeric species (figure 5A). This is consistent with our previous observations (41) that the integrity of cysteine 62 was required for diamide cross-linking of p50: as cleavage II takes place after K77 trypsin cleavage would therefore remove the residues between which disulphides are formed.

As glutaraldehyde is likely to cross-link through multiple residues untreated, form I and form II were briefly exposed to glutaraldehyde, the reaction terminated with sodium borohydride and the products fractionated by SDS polyacrylamide gel electrophoresis under reducing conditions. Cross-linked products were generated from all three species (figure 5B) although at the protein concentrations employed higher order products are also formed. However under these conditions form II molecules can be crosslinked with glutaraldehyde suggesting that they are still dimeric.

Effect of bound DNA on trypsin accessibility of p50

To investigate whether binding of DNA could influence protease accessibility, p50 was incubated with a range of concentrations of trypsin in the presence or absence of double stranded synthetic oligonucleotides containing either high affinity or low affinity κB motifs. Analysis of the cleavage products by SDS polyacrylamide gel electrophoresis revealed that p50 digested in the absence of DNA gave the expected pattern of cleavage with the appearance of form I molecules at low trypsin concentration and the appearance of form II molecules at higher trypsin concentrations. This pattern of cleavage was significantly altered by the presence of DNA containing high affinity κB motifs (H2TFI and NF-κB, reference 45): while the amount of trypsin required for the initial cleavage at R362 to generate form I molecules was unaltered by the presence of DNA the second cleavage at K77 required to generate form II molecules was completely blocked by bound DNA (figure 6A). Although not completely inhibited the cleavage at K77 was severely impaired even in the presence of DNA containing a mutated κB motif (SV1.M1, reference 45) that represents a relatively low affinity binding site for p50 (figure 6A).

As a means of discriminating between the effect of DNA containing either a high affinity κB motif (H2TF1), low affinity κB motif (SV1.M1) or non-specific binding site (poly dA.T, poly dG.C) these DNA species were allowed to interact with p50, exposed to a large amount of trypsin (1:3 ratio of trypsin:p50) and samples removed at various times for analysis on SDS polyacrylamide gels. In the absence of DNA the large amount of trypsin (seen as a stained band in the gel) completely degrades p50 so that after 10 minutes incubation neither form I nor form II molecules are detected. Inclusion of DNA which is bound non-specifically by p50 has only a marginal effect on the rate of trypsin cleavage and again neither form I nor form II molecules are detected after 10 minutes digestion. In the presence of a high affinity κB motif (H2TF1) the resistance of p50 to trypsin cleavage is dramatically increased with form I molecules still predominating after 120 minutes indicating that cleavage at K77 and other basic residues is blocked by bound DNA. Although binding of DNA containing a low affinity mutated κB motif

(SV1.M1) affords p50 some protection from trypsin digestion it is clearly much less efficient in this respect than the DNA containing the high affinity binding site (figure 6B). Thus p50 is protected from trypsin digestion by DNA in relation to the affinity with which it is bound. Although bound DNA could act by simply masking critical cleavage sites it seems likely that bound DNA induces a conformational change in the structure of the protein that renders it resistant to trypsin digestion.

DISCUSSION

The subdomain structure of the p50 subunit of NF-κB has been investigated by partial proteolysis and has revealed that the protein contains a dimeric core that is relatively protease resistant with N and C-terminal extensions that are susceptible to proteolysis. At the C-terminus the nuclear localisation signal (39) occupies a highly exposed position as would be expected of a region that is recognised by the nuclear transport machinery. The nuclear localisation signal also appears to be an important determinant of the interaction between p50 and the inhibitory proteins (IκBs) containing ankyrin repeats (35, 36). At the N-terminus cleavage after K77 removes the terminal 43 amino acids leaving the remainder of the protein devoid of DNA binding activity and suggesting that this region is required for interaction with DNA. This conclusion is strengthened by the observation that the cleavage after K77 did not take place if the protein was first bound to DNA containing its recognition site. This may be due to simple masking of the cleavage site by the bound DNA or it may be that DNA binding is accompanied by a conformational change in the structure of the protein. Although the sites of cleavage were not determined a similar conclusion was reached by Fujita *et al.*(22) who also observed that the sensitivity of the p50 protein to digestion with chymotrypsin was dependent on the precise sequence of the bound DNA. The experiments that were conducted in the presence of large amounts of trypsin suggest that a conformational change may indeed take place. In the absence of DNA the p50 protein was completely degraded, indicating that many cleavage sites were accessible to trypsin. However in the presence of DNA the whole protein is remarkably resistant to cleavage indicating that the sites which were previously accessible to trypsin were now inaccessible. Given the size of the oligonucleotides (about 20 base pairs) it is unlikely that the whole protein could be directly protected from cleavage by the oligonucleotide. One possibility however is that cleavage of the peptide backbone after K77 could destabilise the structure of the entire protein and by blocking cleavage of this key residue bound DNA protects the whole protein from proteolysis. However it is more likely that DNA binding induces an alteration in the structure of the p50 protein. This may be similar to the situation described for the arm of the λ repressor (46, 47) and the β strand motif of the arc repressor (48) where the module that contacts DNA is unfolded in solution but adopts a stable conformation when bound to DNA. A good example from the eukaryotic world of this type of behaviour is provided by the basic region leucine zipper (bZIP) containing proteins GCN4 and fos/jun. In both cases the basic region was largely unstructured in solution but when bound to DNA an α helical structure complementary to the binding site is stabilised (49–56).

A considerable body of evidence suggests that determinants of *rel* protein DNA binding specificity are located between P43 at the start of the *rel* homologous region and the trypsin cleavage site at K77. Mutational analysis has indicated that mutations between R57 and L70 modify the DNA binding properties of the proteins (41, 57, 58) and chemical modification experiments have indicated that bound DNA protects C62 from carboxymethylation by iodoacetate (42). Although secondary structure prediction programmes suggest that this region could form a β strand and it is known that NF-κB proteins make base and backbone contacts over one complete turn of the DNA double helix (45, 59) precise details of the DNA recognition process will only emerge from structural studies.

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