I κ B γ Inhibits DNA Binding of NF- κ B p50 Homodimers by Interacting with Residues That Contact DNA

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NF- κ B is an inducible transcription factor that activates many cellular genes involved in stress and immune response and whose DNA binding activity and cellular distribution are regulated by I κ B inhibitor proteins. The interaction between NF- κ B p50 and DNA was investigated by protein footprinting using chemical modification and partial proteolysis. Both methods confirmed lysine-DNA contacts already found in the crystal structure (K-147, K-149, K-244, K-275, and K-278) but also revealed an additional contact in the lysine cluster K-77-K-78-K-80 which was made on an extended DNA. Molecular modelling of such a DNA-protein complex revealed that lysine 80 is ideally placed to make phosphate backbone contacts in the extended DNA. Thus, it seems likely that the entire AB loop, containing lysines 77, 78, and 80, forms a C-shaped clamp that closes around the DNA recognition site. The same protein footprinting approaches were used to probe the interaction of p50 with the ankyrin repeat containing proteins I κ B γ and I κ B α . Lysine residues in p50 that were protected from modification by DNA were also protected from modification by I κ B γ but not I κ B α . Similarly, proteolytic cleavage at p50 residues which contact DNA was inhibited by bound I κ B γ but was enhanced by the presence of I κ B α . Thus, I κ B γ inhibits the DNA binding activity of p50 by direct interactions with residues contacting DNA, whereas the same residues remain exposed in the presence of I κ B α , which binds to p50 but does not block DNA binding.

Many processes in living cells are regulated by a complex system of gene expression based on the control of transcriptional initiation at the promoter of a gene. Initiation of transcription requires the assembly of a large nucleoprotein complex consisting of DNA, RNA polymerase holoenzyme, and various upstream activators. The frequency of transcriptional initiation can be controlled by the availability or activity of components of the complex, but in many cases the DNA binding upstream activators are the limiting factors responsible for specific gene expression. One such upstream activator is the inducible transcription factor NF- κ B, first isolated as a B-cell-specific DNA binding activity recognizing the 11-bp κ B site in the κ immunoglobulin light-chain enhancer (65) but now recognized to be a ubiquitous factor. NF- κ B regulates the expression of a wide variety of cellular genes involved in the response to infection, injury, or stress and is likely to play a role in growth control and differentiation. In addition, it is an important element in the transcription of viral genes such as those of human immunodeficiency virus (24, 47).

NF- κ B is a heterodimer typically composed of two subunits, p50 and p65 relA (6), belonging to the NF- κ B/rel/dorsal family of transcription factors (7, 66, 69). Members of this family are divided into two groups based on differences in structure, function, and synthesis. The first group includes p50 (22, 36) and p52, which are synthesized as the large precursors p105 and p100, respectively (11, 55, 63), while the second group consists of proteins with a C-terminal transactivation domain (64), including relA (56, 60), relB (61), the proto-oncoprotein c-rel (71), v-rel, and the *Drosophila melanogaster* proteins dorsal

(67) and dif (33). All family members have an approximately 300-amino-acid rel homology region that contains the sequences responsible for DNA binding, dimerization, and nuclear localization. It appears that almost all combinations of homo- and heterodimers can exist and that each of these combinations of proteins prefers certain κ B binding sites and is more susceptible to inhibition by some I κ B proteins than by others. Besides being regulated by combinatorial diversity of dimers, NF- κ B-mediated responses are also controlled by nuclear localization, proteolytic processing, and phosphorylation (43, 69).

In unstimulated cells, NF- κ B proteins are held in the cytoplasm in a form that is unable to bind DNA because they are complexed with inhibitory proteins from the I κ B family (4, 5). Exposure of cells to a wide variety of external stimuli (such as bacterial lipopolysaccharide, phorbol ester, or cytokines) results in the phosphorylation and subsequent degradation (8, 13, 18, 31, 52, 58, 68) of the I κ B protein by the ubiquitin-proteasome pathway (14, 57). The released NF- κ B dimer translocates in an active form to the nucleus and binds to κ B motifs in various target genes. NF- κ B recognizes its binding site in an unusual way involving base and backbone contacts with DNA over one complete helical turn (16, 17). Instead of being made by one specific structural motif (like an α -helix), contacts between the protein and DNA are made in both domains by several loops that are connected by β -strands (21, 54). DNA binding induces a conformational change in NF- κ B (30, 49), presumably a movement of the N-terminal domain at the flexible hinge.

The I κ B family proteins bind specifically and reversibly to NF- κ B proteins (23). This interaction is mediated, at least in part, by the six to eight ankyrin repeats which were first recognized in cell cycle proteins (12) and in erythrocyte ankyrin (46). The integrity of these repeats and the C-terminal acidic domain of the I κ B proteins is important for interaction with, and inhibition of the DNA binding activity of, the correspond-

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ing NF- κ B proteins (10, 29, 32). Ankyrin repeats are also found in the C-terminal regions of the large precursors of the p50 and p52 proteins but are removed by processing via the ubiquitin-proteasome pathway to produce the mature DNA binding forms (57). I κ B proteins show distinct specificity for the various NF- κ B protein dimer combinations. Whereas I κ B γ (C-terminal region of p105) preferentially binds to and inhibits p50 homodimers (44), I κ B α binds to both p50 homodimers and p50-p65 heterodimers but inhibits only the latter (9, 28). Different I κ B proteins are also the final targets of various signalling pathways, depending on the external stimulus. Inducers that cause a transient activation of NF- κ B affect only I κ B α , whereas inducers of a persistent activation appear to target both I κ B α and I κ B β (70). Furthermore, NF- κ B and I κ B α regulate each other through a novel autoregulatory feedback loop in which nuclear NF- κ B induces synthesis of I κ B α mRNA (13, 15, 19, 39, 68). Resynthesized I κ B α translocates to the nucleus, binds to the NF- κ B-DNA complex, and helps to shut down the NF- κ B response (1, 72).

The contacts between I κ B and NF- κ B proteins and the precise mechanism by which I κ B proteins inhibit both the DNA binding activity and the nuclear localization of NF- κ B are still not fully understood. Deletion analysis, antibody accessibility experiments, and protease sensitivity studies have strongly pointed at the nuclear localization signal in the rel homology domain as an area where I κ B α binds to p65 and I κ B γ binds to p50 (9, 48, 51).

The objective of this work was therefore to investigate the interaction of the p50 homodimer with I κ B α and I κ B γ , using a protein footprint technique and partial proteolysis. While footprinting techniques are routinely used in mapping the topography of nucleic acids in nucleoprotein complexes, it is more difficult to apply this approach to proteins because an end-tagged polypeptide (42), a sequence-specific endoprotease, and a reagent that can modify proteins in their native state are necessary. We used a modified version of a footprint technique combining reversible and irreversible lysine modifications in a two-step procedure (26).

By these methods we could confirm the lysine contacts between p50 homodimer and κ B binding sites already found in the crystal structure (21, 54). An additional contact in the lysine cluster (Lys-77–Lys-78–Lys-80) could be found for a 16-mer κ B binding site, supporting the idea of this loop as a C-shaped clamp interacting with the double helix. In the presence of I κ B γ , p50 showed basically the same protection of lysines as was shown with DNA in the footprint experiments, whereas no protection was observed with I κ B α . Proteolysis studies also revealed different conformational states of the p50 homodimer depending on the type of I κ B protein bound.

MATERIALS AND METHODS

Expression and purification of proteins. Expression and purification of a modified version of p50 (p50 ctag) containing the epitope tag GKIPNPLLG LDST (27) fused to its C terminus were as described previously (50, 59). I κ B α , expressed as a glutathione *S*-transferase fusion protein in *Escherichia coli* JM101, was purified by affinity chromatography on glutathione agarose, cleaved with thrombin, and isolated by ion-exchange chromatography on Q-Sepharose as described previously (34, 51). I κ B γ , amino acids 537 to 809 of p105 (36), was expressed in *E. coli* as part of a fusion protein with glutathione *S*-transferase and purified in a fashion similar to that described by Matthews et al. (51), although ion exchange was performed on a 1-ml Mono-Q column (Pharmacia) rather than on Q-Sepharose. The purified proteins were stored in small fractions at -70°C .

Oligonucleotides. Oligonucleotides (12-mer and 16-mer) based on the high-affinity H2TF1 κ B binding site (49) were synthesized on an Applied Biosystems model 381A DNA synthesizer. Known amounts of complementary, single-stranded, synthetic oligonucleotides were annealed by heating to 100°C in a solution containing 0.1 M NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA, followed by slow cooling to room temperature. Double-stranded DNA was precipitated with ethanol and dissolved in a solution containing 20 mM sodium

phosphate (pH 8.3) and 80 mM NaCl, and the concentration was determined on the basis of the A_{260} .

Protein footprinting. The protein footprinting procedure was modified from the original method of Hanai and Wang (26). Prior to reaction with citraconic anhydride (CA), all proteins were dialyzed against a solution containing 20 mM sodium phosphate (pH 8.3) and 80 mM NaCl at 4°C (2). Each reaction mixture (20 μl) contained 1.875 μM p50 ctag and 4 μM either double-stranded DNA, I κ B α , I κ B γ , or bovine serum albumin (BSA). Interaction of p50 ctag with DNA was carried out for 10 min at 20°C , while interaction of p50 ctag with I κ B protein was carried out for 15 min at 37°C . Just before use CA (Pierce) was rapidly diluted to 0.5 mM by the addition of 4.5 μl of the reagent to 100 μl of 20 mM sodium phosphate (pH 8.6) with vigorous stirring at room temperature. The diluted reagent was immediately added to a set of reaction mixtures to give a CA/lysine molar ratio between 0 (control) and 0.5, and the mixtures were held at room temperature for 30 min. The reaction was quenched by addition of an excess of Tris-HCl buffer (pH 8.5). Ice-cold acetone was added to 70%, and the proteins were precipitated at 4°C . The air-dried pellet was dissolved in 100 μl of 7 M guanidinium chloride, buffered with 20 mM sodium phosphate to pH 8.4, vortexed several times, and incubated for at least 45 min at room temperature. To the unfolded peptides, 20 μl of 20 mM sodium phosphate (pH 8.2) and 25 μl of freshly prepared 2% *N*-hydroxysuccinimide acetate (Sigma)-dioxane were added to acetylate irreversibly all lysines (41) that were not citraconylated. The reaction took place for 30 min at room temperature and was again stopped with an excess of Tris-HCl buffer (pH 8.5). Citraconylated lysines were deblocked by dialysis of the reaction mixtures against 30% acetic acid in a microdialysis system (Bethesda Research Laboratories) for 22 h at room temperature (35). Acetic acid in the recovered samples was completely removed under reduced pressure in a spinning evaporator. Protein pellets were dissolved in 10 μl of freshly prepared, deionized 8 M urea for at least 30 min at room temperature. Tris-HCl (pH 8.5) was added, and the sample was diluted to obtain final concentrations of 2 M urea and 0.1 M Tris-HCl. The modified polypeptides were digested with endoproteinase Lys-C (Sigma and Promega), added at a proteinase/protein ratio of 1:200 (wt/wt). The mixture was kept at 37°C for 20 h before analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting).

Partial proteolysis. Purified p50 ctag, in a solution containing 10 mM sodium phosphate and 60 mM Tris-HCl (pH 8.5), was digested with endoproteinase Lys-C (Sigma and Promega) under the conditions indicated in the figure legends. Reactions were terminated by the addition of phenylmethylsulfonyl fluoride followed by heating at 100°C for 2 mins in the presence of SDS and mercaptoethanol prior to analysis by SDS-PAGE and Coomassie blue staining. For analysis by Western blotting, 0.1 volume of the preparation was diluted fivefold and then treated as described above. Partial proteolysis in the presence of DNA was conducted in the same buffer with either a four- or a fivefold molar excess of double-stranded synthetic oligonucleotides (high-affinity κ B motif, 16-mer) over p50 ctag homodimers. Digestion products were analyzed as described above.

Proteolysis experiments with I κ B γ and I κ B α were performed in a solution containing 15 mM Tris-HCl (pH 8.0), 80 mM NaCl, and 10 mM sodium phosphate (pH 8.3). Each reaction mixture contained 2.3 μM p50 ctag homodimer and a fourfold molar excess of the I κ B proteins over the dimer or an equivalent amount of BSA (protease free; Sigma). Samples were digested after 20 min of incubation at 37°C with an endoproteinase Lys-C/protein ratio of 1:300 (wt/wt) and analyzed as described above.

Gel electrophoresis and Western blotting. After being boiled in SDS and mercaptoethanol, protein samples were analyzed by electrophoresis in discontinuous 12.5% polyacrylamide gels containing SDS (38). Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Hybond; Amersham) with a semidry blotter. Membranes were blocked at room temperature in blocking buffer (phosphate-buffered saline [PBS], 0.1% Tween 20, 5% nonfat milk), treated with primary antibody (SV5-PK, diluted 1:1,000) in the same buffer, and then washed with PBS–0.1% Tween 20. Detection of immobilized antigen-antibody complexes was performed with horseradish peroxidase-conjugated anti-mouse immunoglobulins (Amersham), diluted 1:3,000 in blocking buffer, with an enhanced chemiluminescence (ECL) detection reagent (Amersham), and the complexes were visualized on X-ray film (Fuji). The primary antibody used was purified monoclonal antibody SV5-PK, which recognizes the epitope tag in p50 ctag (27). The binding of p50 to ^{32}P -labelled DNA was assessed in a gel electrophoresis DNA binding assay as described previously (48).

Identification of fragments. Peptide length markers for the footprint experiments were generated by partial degradation of the immunotagged p50 either with cyanogen bromide (Sigma), which cleaves polypeptides at methionines (25), or with endoproteinase Lys-C (Sigma), which cleaves polypeptides after lysines. Fragmented polypeptides were modified by acetylation of lysines with *N*-hydroxysuccinimide acetate (Sigma) (42) to directly compare their electrophoretic mobilities with those of the immunotagged peptides from the footprint experiments. This allowed an estimation of fragment sizes and therefore positions of modified lysines. Tricine gels were used for high-resolution separation of fragments, especially for analysis of fragments smaller than 10 kDa, by the procedure of Schagger and Jagov (62), adapted to Bio-Rad minigels. The identities of proteolytic fragments were determined by amino-terminal sequencing. SDS–12.5% polyacrylamide gels were prepared with freshly made acrylamide-piperazine diacrylamide solution (37.5:1, wt/wt), and sodium thioglycolate was added to

FIG. 1. Use of chemical modification to identify lysines at the p50-DNA interface. Purified p50 ctag (1.875 μ M), either free (–) or bound (+) to a double-stranded oligonucleotide containing a high-affinity binding site for NF- κ B, was treated with the indicated ratio of CA to total lysines. After irreversible modification of all remaining lysines and deblocking of citraconylated residues, the protein was digested exhaustively with endoproteinase Lys-C. Digestion products were fractionated either on a standard 12.5% polyacrylamide gel containing SDS (A) or on a Schägger and Jagov (62) Tricine gel (B) and electrophoretically transferred to PVDF membranes, and the tagged protein fragments were immunochemically detected by ECL with the tag-specific monoclonal antibody SV5-PK (27). Fully acetylated p50 ctag cleaved with CNBr was used to provide molecular weight markers (at the left in panel B).

0.1 mM in the upper electrophoresis buffer. Separated fragments were electrophoretically transferred (2 h at 225 mA) onto PVDF membranes (Problot; Applied Biosystems) as described by Jaffray et al. (34). Membranes were washed with distilled water for a few seconds and then stained with 0.1% amido black. The amino-terminal sequence was determined in a Procise microsequencer (Applied Biosystems), with on-line phenylthiohydantoin analysis by Graham Kemp and Paul Talbot, University of St. Andrews.

Molecular modelling. The atomic coordinates of the p50 homodimer bound to DNA (54) were obtained from S. Harrison and R. Chopra, Harvard University. By using the existing DNA duplex, four additional base pairs were added to extend the helix at one end. The structure of the protein was not altered, but inspection of the C-terminal region of the AB loop revealed that K-80 was ideally placed to make contact with the phosphate backbone of the DNA. Figures 4 and 7 were prepared with RASTER3D (3, 53).

RESULTS

Use of chemical modification to identify lysine residues at the p50-DNA interface. To identify lysine residues in the p50 subunit of NF- κ B that are present at DNA-protein and protein-protein interfaces, a recently described (26) two-step lysine modification procedure was employed. As the three-dimensional structure of p50 bound to DNA has been determined (21, 54), the accuracy of data obtained by the chemical modification procedure in the presence and absence of bound DNA can be directly evaluated by comparison with data obtained by crystallographic analysis. In the two-step lysine modification procedure an epitope-tagged protein, either in the absence or in the presence of bound ligand, is exposed to low concentrations of CA, which reversibly modifies exposed lysine residues. The protein is then completely unfolded, and unmodified lysines are irreversibly acetylated in the presence of *N*-hydroxysuccinimide acetate. After acid deblocking of citraconylated lysines the protein is exhaustively digested with endoproteinase Lys-C, which cleaves only at unmodified lysine residues. Analysis of the cleavage products by SDS-PAGE, followed by Western blotting with the tag-specific monoclonal antibody, allows sites of lysine modification to be deduced from the molecular weights of the immunoreactive fragments and the known positions of lysines in the protein. Bound ligand, which blocks the initial, reversible modification at specific lysine residues, will result in these residues being irreversibly acetylated and thus refractory to cleavage with endoproteinase Lys-C. The CA therefore acts as a footprinting reagent which identifies lysine residues displaying altered reactivities in free and bound proteins.

A previously described version of p50 containing a C-terminal epitope tag (59) which is recognized by the SV5-PK monoclonal antibody (27) was produced in bacteria and purified to homogeneity. Addition of the epitope tag to p50 did not change the DNA binding and I κ B γ binding properties of the

protein (data not shown). p50 ctag alone or p50 ctag bound specifically to double-stranded DNA was treated with a range of concentrations of CA such that, on average, less than one modification per p50 ctag molecule was introduced. In the absence of CA the p50 ctag protein is completely resistant to cleavage with endoproteinase Lys-C, indicating that secondary, irreversible modification of the denatured protein was complete (Fig. 1A). CA modification in the absence of DNA results in the generation of protein fragments that are detected by the tag-specific antibody. Comparison with the modification pattern obtained in the presence of DNA indicated that a number of lysines are protected from modification by bound DNA (Fig. 1). To define the sites at which these fragments were cleaved, their molecular weights were determined in a high-resolution polyacrylamide gel (62) by comparison with fragments of p50 ctag generated by treating the fully acetylated protein with CNBr (Fig. 1B).

In the absence of DNA, reversible lysine modification takes place at multiple sites. Although unique residues K-244 and K-252 can be identified as sites of modification, the other modifications occur within lysine clusters where restrictions imposed by the resolving power of the gel preclude definite identification. Nevertheless, it is clear that lysines located between residues 77 and 80, 147 and 149, and 275 and 278 are all sites of modification in the absence of DNA. Bound DNA does not protect p50 ctag from modification at K-252, but modification at K-244 and within lysine clusters 77 to 80, 147 to 149, and 275 to 278 is efficiently blocked. This is in good agreement with structural analysis (21, 54) which has demonstrated that residue K-244 and lysines within clusters 147 to 149 and 275 to 278 are in direct contact with DNA. Although it was inferred that residues in the lysine cluster 77 to 80 might contact DNA outside the 11-bp site, this could not be demonstrated directly, as the cocrystals used for structural analysis were obtained with DNAs containing only 10 or 11 bp (54). In contrast, the protection experiments reported here were conducted with a 16-bp DNA. The abilities of 12- and 16-bp fragments of DNA, which contain the same core sequence, to block lysine modification in the 77-to-80 sequence were therefore directly compared. While the 16-mer DNA blocked modification at 10 μ M, the 12-mer DNA was unable to block modification of lysines located at residues 77 to 80, even at 50 μ M (Fig. 2A). To compare p50 binding to the 12-mer and 16-mer DNA, equivalent concentrations of 32 P-labelled DNA were mixed with a range of concentrations of p50 and DNA-protein complexes were resolved from free DNA in a native polyacrylamide gel. It is clear (Fig. 2B and C) that the 16-mer DNA is bound with a substantially higher affinity than the 12-mer DNA. Thus, the

FIG. 2. Interaction of p50 ctag with 12-mer and 16-mer double-stranded DNA recognition sites. (A) p50 ctag, either free (–) or bound to the indicated double-stranded DNA, was treated with CA and processed for protein footprinting as described in the legend to Fig. 1. Tagged protein fragments were detected by ECL Western blotting with the SV5-PK antibody. The affinity of p50 for the 12-mer (B) or 16-mer (C) double-stranded DNA was determined in a gel electrophoresis DNA binding assay. 32 P-labelled DNA (3 nM) was incubated with a range of concentrations (1.25, 2.5, 3.75, 5, 6.25, 7.5, 8.75, 10, 11.25, and 12.5 nM) of p50, and DNA-protein complexes were separated from free DNA by electrophoresis in a native polyacrylamide gel. B, DNA-protein complexes; F, free DNA.

footprinting procedure has accurately identified those lysine residues in p50 that structural analysis predicts are in contact with DNA. In addition, the footprinting procedure has identified lysines within residues 77 to 80 that are likely to make additional contacts with an extended DNA recognition site.

Probing the p50-DNA complex by partial proteolysis. To independently confirm the results of the lysine modification experiment, an alternative means of probing the accessibility of lysine residues in p50 was employed. p50 ctag, either in the presence or in the absence of DNA, was incubated in the presence of endoproteinase Lys-C, and the cleavage products were fractionated on SDS-polyacrylamide gels which were either stained with Coomassie brilliant blue (Fig. 3A and B) or analyzed by Western blotting with the tag-specific antibody (Fig. 3C and D). In the absence of DNA, p50 ctag is highly susceptible to cleavage with endoproteinase Lys-C (Fig. 3A and C). N-terminal sequencing of membrane-bound protein fragments indicated that the major product had the sequence NKKS_Y. Thus, cleavage of p50 with endoproteinase Lys-C takes place after K-77. At higher protease concentrations, with endoproteinase Lys-C from two different sources, more extensive cleavage of lysine residues which contact DNA is observed, but in both cases this is inhibited by bound DNA (Fig. 3E and F). These data therefore support the conclusion that lysines located within residues 77 to 80 are in direct contact with DNA.

Modelling of p50 bound to an extended DNA recognition site. The preceding data indicate that residues in the lysine cluster located at positions 77 to 80 of p50 interact with an extended DNA recognition site. To establish details of this interaction, atomic coordinates of the p50 DNA cocrystal reported by Müller et al. (54) were obtained from S. C. Harrison (Harvard University). The DNA double helix in this structure was extended at one end by 4 bp of B-form DNA (Fig. 4A), and potential interaction with residues located between 77 and 80 was evaluated. Although the thermal parameters are high, indicating that this region of the protein is not well ordered in the crystal structure, it is clear that without any further manipulation of the structure, K-80 is ideally positioned to contact the phosphate backbone of the DNA (Fig. 4B). In this model the side chain of K-79 is pointed away from the DNA and is therefore unlikely to directly contact the DNA, but relatively small adjustments of the polypeptide backbone could bring K-77 into position to also make contact with the phosphate backbone.

Identification of lysine residues at the p50-I κ B γ interface.

As the two-step lysine modification procedure accurately identified known lysine-DNA contacts in p50 and provided evidence for additional contacts, the same approach was extended to defining the lysine residues in p50 that are protected from modification by interaction with I κ B γ . p50 ctag was incubated in the presence of either BSA or I κ B γ and treated with CA for protein footprinting as described above. In the presence of BSA cleavage took place at those residues at which cleavage was observed when p50 ctag alone was treated with CA (Fig. 5A). In the presence of added I κ B γ many of these cleavages were blocked, but particularly the major cleavage within lysine clusters 77 to 80 and 147 to 149 (Fig. 5A). To illustrate the specificity of this interaction, the protein footprint on p50 which results from interaction with I κ B γ , I κ B α , and DNA was determined. It is evident from the short exposure displayed in Fig. 5B that while both DNA and I κ B γ protect p50 ctag from modification at the lysine cluster 77 to 80, I κ B α does not. The same situation is apparent on the more heavily exposed version of this blot (Fig. 5B), with lysines at 244, 252, and 147 to 149 being protected by I κ B γ but not by I κ B α . Although it is not readily apparent on the blot displayed (Fig. 5A), I κ B γ , but not I κ B α , also protects lysine residues at 275 and 279. Thus, it appears that many of the lysine residues in p50 contacted by DNA are also contacted by I κ B γ , but not I κ B α , even though it has previously been demonstrated that p50 is bound by I κ B α in such a way that the p50 nuclear localization signal is protected from proteolytic cleavage by I κ B α (51).

Probing the interactions between p50 and I κ B proteins by partial proteolysis. To confirm the results obtained by protein footprinting with CA and to obtain information on possible conformational changes induced in p50 by binding of I κ B α and I κ B γ , protein complexes were incubated with endoproteinase Lys-C from two different sources. Digestion products were fractionated in an SDS-polyacrylamide gel and analyzed by Western blotting with the tag-specific antibody. Both forms of endoproteinase Lys-C cleave p50 ctag after K-77. p50 ctag incubated in the presence of BSA and p50 ctag incubated in the presence of I κ B α are cleaved after K-77 at similar rates, but in the presence of I κ B γ the cleavage rate is substantially reduced (Fig. 6). While the rate of cleavage at lysine cluster 147 to 149 is enhanced (relative to the rate in the presence of BSA) by the binding of I κ B α , it is reduced in the presence of I κ B γ (Fig. 6A). To illustrate the sites on p50 thought to be in contact with I κ B γ , the lysine side chains protected from modification with CA and cleavage with endoproteinase Lys-C are displayed in Corey-Paulin-Koltun format on the ribbon dia-

FIG. 3. Partial proteolysis of p50-DNA complexes. p50 tag (25 μ M, 20 μ g), either in the absence (A and C) or in the presence (B and D) of 16-mer DNA (50 μ M), was treated with endoprotease Lys-C (Endo Lys C; 2.5, 5, 10, 20, 40, and 100 ng) from *Lysobacter enzymogenes* (L. enz) for 60 min at 20°C. Reaction products were separated in a 12.5% polyacrylamide gel and either stained with Coomassie brilliant blue (A and B) or processed for ECL Western blotting with the tag-specific monoclonal antibody SV5-PK (C and D). The positions of molecular weight markers are indicated on the left. To increase the extent of digestion, p50 tag (10 μ M, 8 μ g), either in the absence or in the presence of 16-mer DNA (50 μ M), was treated at 37°C with endoprotease Lys-C (40 ng) from either *L. enzymogenes* (E) or *Pseudomonas aeruginosa* (P. aer) (F). Aliquots were removed at the times indicated, and the reaction was terminated with phenylmethylsulfonyl fluoride. Digestion products were separated on a 12.5% polyacrylamide gel and electrophoretically transferred to PVDF membranes, and the tagged protein fragments were immunochemically detected by ECL with the SV5-PK antibody.

gram of p50 (Fig. 7A) derived from the cocrystal structure (54). For comparative purposes the sites in p50 that are protected by bound DNA from cleavage with a variety of proteases (49) are also displayed (Fig. 7B). Although not all of the residues displayed in Fig. 7A are points of DNA contact, it seems likely that I κ B γ interacts with a surface on p50 similar to that occupied by specifically bound DNA.

DISCUSSION

The utility of the lysine modification procedure has been established for footprinting proteins bound to DNA (26), but we have now demonstrated its usefulness for the analysis of protein-protein interactions. Lysines are ideal residues to probe in protein-ligand interactions, as they are abundant in most proteins (there are 28 lysine residues in the p50 polypeptide) and most of them form part of the surface of a protein, exposing their charged (or at least polar) side chains to the environment. The reactivity of the ϵ -amino group of this side chain and the availability of reversible and irreversible modification reagents further recommend these residues for such experiments. As the nucleoprotein or protein-protein com-

plexes are treated only lightly with the reversibly modifying agent, structural perturbations of these complexes are minimized.

As the positively charged lysine residues in p50 that contact bound DNA were identified by X-ray crystallography (21, 54) during the course of this work, the results of the footprinting could be used to confirm these contacts and to show possible additional contacts. The X-ray crystallographic analysis (21, 54), spectroscopic studies (49), partial proteolysis experiments (30), and footprint results greatly complement and confirm each other and together give a detailed account of the dynamic interaction between NF- κ B and its DNA binding site.

We have interpreted the DNA-mediated protection observed in the C-terminal region of the AB loop at K-77, K-78, and K-80 as contacts between these residues and the phosphate backbone of DNA. While such a contact was inferred from the crystallographic studies, this was not directly demonstrated, as the DNA in the cocrystals contained only a continuous duplex of 11 bp. Interactions unique to the crystal lattice determined the conformation of the DNA at the molecular ends, which are not part of the continuous duplex (54). How-

FIG. 4. Modelling the interaction of p53 with an extended DNA. By using atomic coordinates of the p53-DNA cocrystal obtained by Müller et al. (54), the DNA was extended by four GC base pairs (in brown). Only a single subunit of p53 (blue at the N terminus, changing to red at the C terminus) is shown interacting with the extended DNA (A). The C-terminal portion of the AB loop, containing lysine cluster 77 to 80, is shown (B), with the side chain of K-80 located such that it could interact with the DNA phosphate backbone. The model was prepared with RASTER3D (3, 53). *N-ter*, N terminus; *C-ter*, C terminus.

ever, a number of lines of evidence point to elements of this lysine cluster being in direct contact with the DNA. Partial proteolysis experiments reported here and previously (30, 49) identified this region as being resistant to proteolysis in the presence of bound DNA. Cleavages after Y-60, S-74, K-77, and N-78 were all blocked by bound DNA, and while Y-60 is part of the recognition loop responsible for specific DNA binding,

the other residues are all present in the C-terminal region of the AB loop. Although a conformational change, induced by DNA binding, could also make these residues inaccessible to protease, it is unlikely that this would also be the case for the lysine modification reagent CA. Protection of this region was observed only when p53 was bound to a 16-mer or longer oligonucleotide and not when it was bound to a 12-bp oligo-

FIG. 5. Identification of lysine residues in p50 at the interface with I κ B γ . (A) Purified p50 ctag was incubated with either BSA or I κ B γ and treated with CA. Reactions lacking CA were included as controls. After irreversible modification of all remaining lysines and deblocking of citraconylated residues, the protein was digested exhaustively with endoproteinase Lys-C. Digestion products were fractionated on a 12.5% polyacrylamide gel containing SDS and electrophoretically transferred to PVDF membranes, and the tagged protein fragments were detected by ECL with the SV5-PK antibody. A short exposure (s.e.) and a long exposure (l.e.) of the film are shown. The positions of modified lysine residues, determined by comparison with the migration of fully acetylated p50 ctag cleaved with CNBr, are indicated. (B) Direct comparison of lysine residues in p50 ctag modified when the protein is citraconylated either alone (–) or in the presence of DNA, BSA, I κ B γ , or I κ B α . Digestion products were analyzed as described for panel A.

nucleotide that was similar in size to that used for crystallographic analysis. This was reflected in the binding of p50 to these DNA duplexes: the short DNA bound poorly, whereas the larger DNA bound with high affinity. Thus, it seems likely that this region contacts DNA directly. This was also suggested by modelling experiments using the coordinates for the p50-DNA cocrystal, in which the DNA was extended. Without further manipulation of the protein sequence, it was clear that the side chain of K-80 could make good contact with the DNA phosphate backbone. The resolution of the SDS-polyacrylamide gels used in our analysis did not allow us to unambiguously identify this lysine as a DNA contact. The sensitivity of the AB loop in the absence of DNA suggests that it is highly

flexible, and this is borne out by the crystallographic data. These data demonstrate that in the cocrystal the C-terminal region of the loop, which is not in contact with DNA, has high thermal parameters, indicating that it is not highly ordered.

One important mode of regulation of the NF- κ B proteins is interaction with proteins from the I κ B family, because they are responsible for cytoplasmic retention and inhibition of DNA binding. It has been shown previously that I κ B γ occludes the nuclear localization signal of p50 (51), but this information did not indicate how I κ B γ could inhibit the DNA binding activity of the protein. Results presented here indicate that I κ B γ does this by contacting the very loops that p50 uses to contact the phosphate backbone of the DNA. The specificity inherent in this process is illustrated by the observation that while both I κ B α and I κ B γ are known to interact with p50 homodimers, only the latter protects lysines in the DNA binding loops from chemical modification and inhibits DNA binding activity. Thus, it seems likely that I κ B α and I κ B γ have two distinct modes of interaction with p50 homodimers. Both proteins appear to interact initially with the nuclear localization signal of p50 (9, 51), but while the binding of I κ B α is weak and can be reversed by the addition of DNA containing the recognition site for p50, I κ B γ binds tightly, occluding the DNA binding site of p50. A recent mutational analysis of dorsal-cactus interactions carried out with yeast cells (40) identified two dorsal mutations which rendered the protein refractory to inhibition by cactus. By comparison with the known p50-DNA structure, these mutations were mapped to a surface of the protein distinct from that involved in DNA binding but close to the nuclear localization signal. No other dorsal mutations were detected. However, the genetic screen used to isolate these mutants was based upon the mutated dorsal protein being fully competent for nuclear transport, DNA binding, and transcriptional activation. Thus, the investigators did not isolate mutations in the nuclear localization signal and would not have detected mutations in the loops which interact with DNA, as it is likely that these mutations would also disrupt DNA binding. The conclusions reached here are also consistent with previously reported experiments on the analogous interaction between I κ B α and relA, in which bound I κ B α and pp40 occlude regions of the AB loops in relA and v-rel that are predicted to be in contact with

FIG. 6. Partial proteolysis of p50 bound to I κ B α or I κ B γ . p50 ctag mixed with BSA, I κ B γ , or I κ B α was incubated for the times indicated with endoproteinase Lys-C (Endo Lys C) from either *P. aeruginosa* (*P. aer*) (A) or *L. enzymogenes* (*L. enz*) (B). Digestion products were separated on a 12.5% polyacrylamide gel and electrophoretically transferred to PVDF membranes, and the tagged protein fragments were detected by ECL with the SV5-PK antibody. The sites at which the protease cleaves are indicated on the right.

FIG. 7. $\text{I}\kappa\text{B}\gamma$ occupies the same surface on p50 as does specific DNA. (A) The side chains of lysine residues that show altered reactivity to CA or altered accessibility to proteases in the presence of $\text{I}\kappa\text{B}\gamma$ are displayed as space-filled Corey-Paulin-Koltun models on the ribbon representation of a single subunit of p50 (blue at the N terminus, changing to red at the C terminus). The p50 structure shown is derived from the atomic coordinates of the p50-DNA cocrystal obtained by Müller et al. (54), from which the DNA chains have been removed. (B) Side chains of p50 that show altered sensitivity to a variety of proteases (49) are shown space filled for comparison. Details of the structure are as in panel A. The model was prepared with RASTER3D (3, 53).

DNA (20, 37). Although the residues in $\text{I}\kappa\text{B}\gamma$ which contact the DNA binding loops of p50 have not been identified, it is tempting to speculate that this is accomplished by acidic residues present in the C terminus of the protein. Deletion of these sequences from the non-DNA binding p105 (where $\text{I}\kappa\text{B}\gamma$ is contiguous with p50) converts the protein into a form that is capable of binding DNA (10). In $\text{I}\kappa\text{B}\alpha$ the equivalent acidic region appears to represent a distinct domain (34) that is required for inhibition of relA DNA binding but is dispensable for simple interaction with relA (20). Thus, a model for the interaction of $\text{I}\kappa\text{B}$ molecules with DNA-bound rel proteins

would involve initial recognition of the rel protein in the region of the nuclear localization signal to form a ternary complex containing $\text{I}\kappa\text{B}$, rel protein, and DNA. The half-life of this complex would be dependent on the combination of $\text{I}\kappa\text{B}$ molecule, rel protein, and DNA sequence bound, and the complex could decay by release of either $\text{I}\kappa\text{B}$ or DNA (51). Displacement of bound DNA may be mediated by a highly specific interaction between the acidic C-terminal domain of the cognate $\text{I}\kappa\text{B}$ protein and the bound rel protein. This mechanism of action is remarkably similar to that of the *Bacillus subtilis* δ factor, which contains a highly acidic region thought to be directly responsible for the displacement of RNA from RNA polymerase (45).

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