The Structure of the Nuclear Factor-κB Protein-DNA Complex Varies with DNA-binding Site Sequence*

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Transcriptional regulation of many immune responsive genes is under the control of the transcription factor NF-κB. This factor is found in cells as a dimer which can contain any two members of the Rel family of proteins (p50, p65, p52, c-Rel, and RelB). The different dimers show distinct preferences for DNA-binding site sequences. To understand the relationship between the DNA binding properties of the dimer forms and transcriptional activation, the physical properties of the complexes of p50 and p65 with DNA have been analyzed. Comparison of apparent DNA binding affinity showed differences in selectivity of DNA-binding site sequence. The ionic strength dependence of apparent binding affinity has shown that the number of ionic interactions in the protein-DNA complex depends on the DNA-binding site sequence and the dimer form, which are consistent with changes in the structure of the protein-DNA complex. Using a fluorescent technique to measure DNA structure changes, protein binding does not appear to alter the structure of the DNA-binding site within the limits of detection. These results are consistent with a change in protein structure that may result in activation differences due to alternative interactions with other transcription proteins.

The transcription factor NF-κB has been shown to activate transcription from many genes involved in the immune response (for review, see Ref. 1) and from several viral promoters (HIV1 (2) and cytomegalovirus (3)). The central role for NF-κB in inflammation has been confirmed in mice lacking the p50 or the RelB subunit of NF-κB. These animals show severe alterations in their response to immune challenge (4, 5). In addition, p65-deficient B- and T-cells show severe reductions in cellular activation (6). The active DNA binding form of NF-κB is a dimer and can consist of any combination of five different NF-κB/Rel protein subunits (p50, p52, p65, c-Rel, and RelB) (1, 7). Many previous studies have shown that dimers of the different monomeric forms show differences in sequence selectivity. Early studies looking at p50 and p65 DNA binding specificity suggested that the p65 subunit extended the specificity of p50 (8). In studies of DNA-binding site selection, Kunsch et al. (9) have shown defined selectivities for each dimer form using electrophoretic mobility shift assays. Perkins et al. (10) have shown that subunit composition could influence transcriptional activation in transfection assays in Jurkat T-cells. It has also been shown that the 3′-half site of a NF-κB element has a profound effect on the size of NF-κB dimer that binds to that site (11). Finally, Schmid et al. (12) have shown that the p50 and p49 subunits of NF-κB have distinct binding properties and function differently on different DNA-binding sites. Thus, there is clear evidence that there are differences between the protein-DNA complexes that depend on the dimer form and the DNA sequence in the complex. However, there have been few studies to identify and characterize what the structural differences could be between these complexes.

The structures of the p50, p52, p65 homodimer, and p50/p65 heterodimer protein-DNA complexes have been reported (13–17) and have provided a great deal of insight into the mechanisms of specificity of these protein-DNA interactions. It is clear that there are many direct contacts between the protein and DNA in the DNA-binding domain that afford specificity to a consensus DNA-binding site. However, there are still many questions about how the observed structures relate to transcriptional activity. Although the structures reported show differences in the DNA-binding domain, it is unclear if there are any changes in the DNA-binding domain when the protein is bound to different DNA-binding sites. These structures were solved with a limited number of DNA sequences, so any changes in complex structure that depend on the DNA sequence in the site cannot be defined. Understanding these changes in structure are particularly interesting as the residues that interact with the DNA in a sequence specific manner are found in a portion of the protein that is likely to be flexible owing to its location at the end of a β-sheet structure (14). Because, the differences in activity observed when bound to alternate sites is likely to be biologically significant, we have analyzed the physical characteristics of p50/p50, p65/p65, and p50/p65 dimer binding to several DNA-binding site sequences. Using both nitrocellulose filter binding and fluorescence polarization, the apparent dissociation constants for protein-DNA site interactions were determined under various conditions. This analysis has identified several interesting aspects of the NF-κB-DNA complexes. The data show that the p50 homodimer can bind to nonspecific DNA with a higher apparent affinity than the p65 homodimer. Also, the NF-κB-DNA interaction is sensitive to the anion in the buffer, which suggests that ions are released from the protein upon binding. Finally the number of ions released upon complex formation is dependent on the DNA sequence of the binding site and the dimer form. This last result is consistent with the hypothesis that the conformation of the protein-DNA complex can vary with DNA-binding site sequence.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The GSTp50 and GSTp65 prokaryotic expression plasmids were a generous gift of Drs. Neal Perkins and Gary Nabel (18). GST fusion proteins were expressed and purified

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Table I

**NF-κB site sequence variants used in this study**

| Binding sites | Sequence |
|---------------|----------|
| Hu Ig-κ       | GGGGATTC |
| β-Interferon   | GGGAATTC |
| VCAM-63       | GGGAATTC |
| VCAM-77       | GGGTATCC |
| p50 consensus | GGGATTCG |
| Hu Ig-κ mutant 1 | GGGATTC |
| Hu Ig-κ mutant 2 | GGGAATTC |
| HIV LTR       | GGGAATTC |
| Mutant        | GGGATTC |
| TATA          | GCGAGAG |

Flanking sequences

- 20-mer: GCCCT... ... TTGAA
- 18-mer: CCCT... ... TTGA

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Thrombin, 50 μg/ml bovine serum albumin and indicated concentration of NaCl or sodium acetate) at 37 °C. Labeled binding site was added to 0.5 ml of buffer in 10 × 75-mm tubes and the initial polarization reading was taken. Protein aliquots were then added to the tube and additional readings were taken until the readings did not change (within 2 min) and the last 2 readings were averaged in the data shown. The data from these experiments was plotted and fit to a hyperbola using the nonlinear curve fitting functions of Origin analysis software package (MicroCal, MA).

**RESULTS**

Different NF-κB Forms Show Different Selectivity for DNA-Binding Sites—Previous studies have shown that the alternative dimer forms prefer different DNA-binding site sequences (9) that can effect transcriptional activation (10). We have analyzed the relative affinities of several DNA-binding sites for p50/p50, p65/p65, or p50/p65 using purified fusion proteins and defined flanking sequences. Fig. 1 shows data for p65 homodimer and 3 different competitor DNA-binding sites. The data have been normalized to the concentration required to inhibit binding to 50% of the highest affinity competitor (Table II). As expected from previous studies, the order of apparent affinities for p50 homodimer is different from that of p65 homodimer. The apparent affinity for p65 homodimer binding to any of these sites varies over a 30-fold concentration range, while the range of apparent affinity for p50 homodimer with the same DNA-binding sites is less than 10-fold. The p50/p65 heterodimer order of apparent affinity is similar to that of p65, however, the range of apparent affinities for the p50/p65 heterodimer is smaller than that of p65 homodimer. Thus, in this

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set of binding sites, the p65 monomer seems to define the selectivity for the p50/p65 heterodimer complexes. Also, the smaller difference in apparent affinity observed for p50 homodimer, compared with p65 homodimer, suggests that p65 binding is more sensitive to DNA-binding site sequence than p50, for the DNA-binding sites tested.

The Apparent Affinity of the p50 Homodimer for Nonspecific DNA Is Higher Than Observed for the p65 Homodimer—The filter binding assay is useful for surveying many different sites, but a solution binding assay is preferred for detailed binding studies. Fluorescence polarization has been used to study protein association with fluorescently labeled oligonucleotide-binding sites. Labeled DNA alone shows low fluorescence polarization in solution (typically ~60–80 millipolarization units). Upon addition of protein, the polarization increases to ~180–200 millipolarization units for the protein-DNA complex. Previous experiments in this laboratory have shown that the cellular activity of the human Igκ, β-interferon, and the HIV NF-κB DNA-binding sites are very different depending on the dimer composition, so these sites were chosen for examination. As shown in Fig. 2A, increasing the protein concentration increases the observed fluorescence polarization. This change can be observed for the three forms of NF-κB that have been tested (p50/p50, p50/p65, and p65/p65). For each site the starting polarization value of free DNA was constant, however, the three sites did show slightly different polarization in the absence of protein (Igκ = ~95, HIV = ~90, and interferon = ~110). The polarization value of free DNA-binding site has been subtracted from each data point for analysis. The data from the filter binding experiments showed that there was less of a change in affinity with binding site for p50 than observed for p65. This observation can be interpreted to suggest that p50 is less binding site selective than p65. Since selectivity is related to the difference between the affinity of the protein to nonspecific sites compared with that for specific sites, the ability of p50 homodimer and p65 homodimer to bind to an oligonucleotide that does not contain a consensus NF-κB-binding site was measured. As shown in Fig. 2B, the fluorescence polarization of the nonspecific DNA-binding site increased with increasing concentration of p50 homodimer. A small change was observed only at high concentrations of p65 homodimer, suggesting that, under these conditions, p50 homodimer shows a higher apparent affinity for nonspecific DNA than p65 homodimer. These data can be compared with binding to a specific site and under the conditions tested, the difference between the binding affinity of p50 homodimer for a nonspecific

| Site         | Sequence          | Relative apparent affinity |
|--------------|-------------------|----------------------------|
|              | p65               | p50/p65                    | p50  |
| β-Interferon | GGGGAATTC      | 1                          | 1    | 9    |
| Hu Igκ mutant 1 | GGGGAATTC  | 4                          | 1    | 6    |
| VCAM-63      | GGGATTC       | 4                          | 2    | 2    |
| Hu Igκ       | GGGGAATTC     | 7                          | 3    | 2    |
| Hu Igκ mutant 2 | GGGGAATTC   | 9                          | 6    | 5    |
| HIV LTR      | GGGCAATTC    | >30                        | 13   | 1    |
| VCAM-77      | GGGTTC       | >30                        | 17   | 3    |
| p50 consensus | GGGATTC     | >30                        | 18   | 2    |

3 J. B. Marine and J. P. Menetski, unpublished observations.
the number of direct ionic interactions and with the specific site data (slope DNA shows a reduced dependence on ionic strength compared

Results were analyzed in a log-log analysis. As shown in Fig.
determined at several sodium acetate concentrations and the

described under "Experimental Procedures." The initial polarization has
been subtracted from each point so that the initial reading is zero to
allow curve fitting.

versus the log monovalent cation is equal to m'ψ, where m' is
the number of direct ionic interactions and ψ represents the
number of thermodynamically bound cations to the polyanion.
The log-log analysis for p50 or p65 homodimer or the p50/p65
heterodimer with the human Igκ, β-interferon, or the HIV
NF-κB DNA-binding site is shown in Fig. 4. The slopes from the
data determined using the interferon (Fig. 4A) and HIV (Fig.
4B) sites are consistently 3–4 (Table III), for all of the protein
forms tested. Interestingly, the human Igκ site (Fig. 4C) shows
a difference in the slopes observed with the p65 homodimer
(3.4) compared with the p50 homodimer or the p50/p65 het-
erodimer (6.0 and 6.1, respectively). These data suggest that
more ions are displaced upon complex formation with p50.
Thus, the complexes formed with the human Igκ site must be
structurally different in the complexes containing p50 com-
pared with those formed with p65 alone.

Because the p50 homodimer can bind to nonspecific DNA
with an appreciable affinity, the ionic strength dependence of
this interaction could also be determined. Apparent DNA bind-
ing affinity of p50 binding for a non-NF-κB-binding site were
determined at several sodium acetate concentrations and the
results were analyzed in a log-log analysis. As shown in Fig.
4D, the apparent affinity of p50 homodimer for nonspecific
DNA shows a reduced dependence on ionic strength compared
with the specific site data (slope = 1.4). These results suggest
that the contacts in the nonspecific DNA complex are not ex-
actly the same as those found in the specific complex. Several
reports have suggested that the anionic component of the
buffer can effect binding affinity. In addition, the anion can also
be found to effect the slope in a log-log analysis, suggesting that
some of the ions displaced upon binding are anions (24, 25).
Analysis of the effect on the anionic component on p50 ho-
modimer binding is shown in Fig. 5, however, similar data have
been obtained for p65 homodimer. Substitution of chloride for
acetate significantly reduces the apparent affinity of p50 ho-
modimer for DNA. However, the slope of the log-log analysis is
the same for chloride or acetate. Thus, although the apparent
affinity of DNA binding is affected the net number of ions
displaced is unchanged.

Binding of p50 or p65 to the β-Interferon or the Human Igκ
Does Not Significantly Effect Bending of the Site as Measured
by Fluorescence Resonance Energy Transfer—Since the ionic
strength dependence data suggest that there is a difference in

FIG. 3. Ionic strength affects apparent affinity of the NF-κB-
DNA complex. Binding experiments are shown for p50/p50 titration of
the β-interferon DNA-binding site at increasing sodium acetate concen-
trations (50 mM, squares; 100 mM, circles; 150 mM triangles) as de-
scribed under “Experimental Procedures.” The initial polarization has
been subtracted from each point so that the initial reading is zero.

In this report, we assess the biochemical characteristics of
NF-κB-DNA complex formation with several different sites.
These analysis has lead to several conclusions. The p50 ho-
modimer shows less selectivity of DNA-binding site sequence
than the p65 homodimer. Also, the ionic strength dependence of
complex formation is dependent on both the NF-κB dimer for
and the DNA-binding site sequence. Finally, the apparent
affinity of the NF-κB protein for DNA is influenced by the
anionic component of the buffer. This type of physical data,
along with models of the protein from x-ray crystallographic
studies is necessary for understanding the determinants of
DNA binding specificity and how they relate to the structure of
the protein-DNA complex.

While studying DNA-binding site selectivity, the interaction
with nonspecific DNA has also been addressed. The data show
that p50 binds to nonspecific DNA with a higher apparent
affinity than p65 under the conditions tested. We can define
specificity of binding to a DNA sequence as the ratio of the
apparent affinity for a specific DNA-binding site sequence di-
vided by the apparent affinity for nonspecific DNA. For p50,
this comparison is complicated by the finding that the ionic
strength dependence for specific and nonspecific binding sites
is different. The data show that the specificity of p50 for a
specific sequence decreases as ionic strength increases, because
nonspecific binding is less sensitive to ionic strength than
specific binding. A comparison of p50 specificity at several
sodium acetate concentrations shows that at low ionic strength
(50 mM) the specificity of binding is between 100 and 4000
depending on the site, but a physiological ionic strength the
specificity is significantly lower (5–10). The significance of this

DISCUSSION

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modimer shows less selectivity of DNA-binding site sequence
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the protein-DNA complex.
result is unclear, however, these experiments were done using short DNA-binding sites (18 nucleotides) and we have observed that the apparent affinity of DNA binding is very sensitive to binding site length (data not shown). It is possible that the apparent affinity dependence on length is a consequence of the nonspecific affinity and is a mechanism used by p50 to find specific DNA-binding sites. Thus, in the context of a longer DNA sequence the apparent affinity of a specific site might increase, which would increase the overall specificity. However, further analysis of this interaction would be required to test this hypothesis.

The selectivity of different forms of NF-κB has been previously described (9). Since the amino acid sequences of the monomers are different, it was assumed that the selectivity arose exclusively from different amino acid-nucleotide base contacts. Recent descriptions of the structures of NF-κB-DNA complexes have shown that many hydrogen bonds are made between the protein monomer and the bases in the DNA-bind-

| DNA site        | p65/p65 | p50/p50 | p50/p65 |
|-----------------|---------|---------|---------|
| Nonspecific     | 1.3     | 3.1     | 2.5     |
| β-Interferon    | 3.0     | 3.4     | 4.0     |
| HIV LTR         | 3.4     | 6.0     | 6.1     |
| Hu Igκ-E-selectin | 3.4     | 6.0     | 6.1     |
reported structures for Rel family proteins (13–16) show that allowing for differences in hydrogen bonding patterns. The NF-κB, or presence of protein (solid line).

addition of p65. Emission spectra are shown in the absence of protein mine (acceptor) to measure changes in distance due to structural TBP and 2 nM DNA-binding site.

FIG. 6. Fluorescence resonance energy transfer (FRET) to assess changes in DNA structure upon NF-κB binding. Oligonucleotide binding sites were 5’-labeled with fluorescein (donor) and rhodamine (acceptor) to measure changes in distance due to structural changes in the DNA. Experiments were done at 10 nM p50, or 35 nM TBP and 2 nM DNA-binding site. A, TBP binding to TATA-binding site. B, NF-κB binding to β-interferon, or C, hu IgK/E-selectin site upon addition of p65. Emission spectra are shown in the absence of protein (solid line) or presence of protein (dashed line).

ing site (13–16). Five amino acids in the p50 monomer appear to make direct hydrogen bonds to bases and two of these are different between p50 and p65 (His$^{67}$ → Ala; and Lys$^{244}$ → Arg) allowing for differences in hydrogen bonding patterns. The reported structures for Rel family proteins (13–16) show that the major DNA recognition amino acids are found on a loop between two β-sheet structures. In the absence of DNA, this loop has been suggested to be flexible since it is not contained in a defined secondary structure motif. These data suggest that the DNA-binding site of Rel family proteins is likely to be flexible enough to allow some movement when associated to DNA-binding site sequences other than those that are found in the crystal structures. Recently, the crystal structure of the p65-DNA complex has been described to have structural differences in the DNA-binding domains of the two monomers (16). The ionic strength dependence of the affinity of a protein for DNA is related to the number of ions released to solvent upon complex formation (22). If the number of ions released changes, it may be related to a change in the structure of the protein. The change in the ionic strength dependence of NF-κB-DNA complex formation described in this report, suggest that the structure of the p50-DNA complex containing either the β-interferon or the hu IgK sites are different. These results are consistent with the structural changes observed in the p65 homodimer crystals. Thus, this data provides physical evidence for flexibility in the DNA-binding site sequence. In addition the data suggests that, along with different amino acid contacts, the structure of the protein may be altered when bound to different DNA-binding site sequences.

Thus, these data are consistent with a model of NF-κB binding to DNA that allows some variations in the DNA-binding domain of the protein to accommodate different DNA-binding site sequences. This model might explain why only some NF-κB DNA-binding sites promote cooperative interactions with other transcription factors or accessory proteins like high mobility group-I(Y) (31). It is also possible that the alternative structures are associated with NF-κB transcriptional activity directly, as some strong p65 homodimer DNA-binding sites are found to be very poor activators of transcription (32). Therefore, the alternative structures of NF-κB complexes may add another level of regulation to transcription by this complex by using the DNA-binding site as an allosteric effector of NF-κB activity.

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