NF- κ B p52:RelB heterodimer recognizes two classes of κ B sites with two distinct modes

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The X-ray structure of the nuclear factor- κ B (NF- κ B) p52:RelB: κ B DNA complex reveals a new recognition feature not previously seen in other NF- κ B: κ B DNA complexes. Arg 125 of RelB is in contact with an additional DNA base pair. Surprisingly, the p52:RelB R125A mutant heterodimer shows defects both in DNA binding and in transcriptional activity only to a subclass of κ B sites. We found that the Arg 125-sensitive κ B sites contain more contiguous and centrally located A:T base pairs than do the insensitive sites. A protein-induced kink observed in this complex, which used an AT-rich κ B site, might allow the DNA contact by Arg 125; such a kink might not be possible in complexes with non-AT-rich κ B sites. Furthermore, we show that the p52:RelB heterodimer binds to a broader spectrum of κ B sites when compared with the p50:RelA heterodimer. We suggest that the p52:RelB heterodimer is more adaptable to complement sequence and structural variations in κ B sites when compared with other NF- κ B dimers.

Keywords: NF- κ B; κ B DNA; RelB; p52; X-ray crystallography

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INTRODUCTION

The nuclear factor- κ B (NF- κ B) family of proteins is an important class of transcription activators that regulates the expression of genes involved in a diverse array of biological functions, including immune and inflammatory responses, cell growth, proliferation and survival (Claudio *et al*, 2006; Hayden *et al*, 2006). There are 12 physiological NF- κ B dimers formed from five subunits; p50, p52, RelA (p65), cRel and RelB. Active NF- κ B dimers bind to specific DNA sites that are present in the promoters and enhancers of target genes, which are collectively known as κ B sites (Hoffmann *et al*, 2006). The DNA-binding segment of these proteins is highly homologous and is referred to as the rel homology region (RHR). The RHR is formed by two folded immunoglobulin-like domains and both domains are involved in

DNA contact. A short 10-residue-long linker connects the two domains and is also directly involved in DNA contact. Although a large number of κ B sites follow the 5'-GGGRNWWYCC-3' consensus, there is a significant number that do not (R = purine, Y = pyrimidine, W = A or T and N = any nucleotide). Several NF- κ B homo- and heterodimers bound to DNA have been solved (Ghosh *et al*, 1995; Muller *et al*, 1995; Cramer *et al*, 1997; Chen *et al*, 1998a,b; Huang *et al*, 2001). These structures show a common DNA-binding mode in which the amino-terminal immunoglobulin-like domain makes base-specific contacts, and the smaller carboxy-terminal domain makes both nonspecific DNA contacts that stabilize the protein–DNA complex and protein–protein contacts that form a dimer.

Two main NF- κ B dimers are the p50:RelA and p52:RelB heterodimers. In response to a class of inducers that includes proinflammatory cytokines, the p50:RelA heterodimer is rapidly activated from the I κ B-bound inhibited state through the degradation of I κ B. Free p50:RelA then activates a large number of target genes by binding directly to their κ B sites. The p52:RelB heterodimer is activated slowly through the processing of p100 into p52 (Senftleben *et al*, 2001; Xiao *et al*, 2001). The processing event requires activation of the NF- κ B-inducing kinase–I κ B kinase 1 signalling cascade in response to a specific class of stimuli (Ghosh & Karin, 2002). The p52:RelB heterodimer is involved in the prolonged activation of NF- κ B target genes involved in developmental programmes in lymphoid cells (Weih *et al*, 2001; Dejardin *et al*, 2002; Yilmaz *et al*, 2003; Claudio *et al*, 2006; Basak *et al*, 2007).

The basis for NF- κ B dimer-specific gene expression is now under intense investigation. *In vitro* experiments have generated some rules on how various dimers bind to κ B sites. However, these rules have failed to explain genetic data clearly, which suggest that, with some exceptions, there is no clear dimer specificity in gene regulation (Hoffmann *et al*, 2003, 2006; Natoli, 2006). It has been reported that the p52:RelB heterodimer binds to and activates a unique class of genes that contain κ B sites that diverge significantly from classical κ B sites. These p52:RelB-specific sequences with consensus 5'-RGGAGAYTTR-3' (R = A or G and Y = C or T) are present in the promoters of four chemokines, stromal cell-derived factor 1 (*SDF1*), B lymphocyte chemokine (*BLC*), Epstein–Barr virus-induced molecule-1-ligand chemokine (*ELC*) and secondary lymphoid organ chemokine (*SLC*), that are

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involved in lymphoid development and the maintenance of splenic architecture (Bonizzi *et al*, 2004). However, a recent study has shown that these chemokines are also activated by the p50:RelA dimer in mouse embryonic fibroblast cells (Basak *et al*, 2008). Surprisingly, another recent report has also shown that the p52:RelB heterodimer does not bind to the κ B sequences, *SDF1*, *BLC*, *ELC* and *SLC* promoters *in vitro* (Britanova *et al*, 2008). Therefore, it is unclear whether discrimination in gene expression by NF- κ B dimers is a direct result of discrimination in κ B DNA binding. In particular, κ B DNA binding by the p52:RelB heterodimer has remained a puzzling issue.

The focus of this study was to investigate the mechanism of DNA recognition by the p52:RelB heterodimer. The constructs used and the purity of the complex used for crystallization are shown in supplementary Fig 1 online. We report the 3.05 Å crystal structure of p52:RelB RHR heterodimer bound to a κ B DNA of sequence 5'-CGGGAATCCCC-3' (supplementary Table 1 online). Structure-based mutational analysis allows us to propose a model of how the p52:RelB heterodimer would be able to bind to a large variety of κ B sites and activate a broad spectrum of genes involved in both inflammatory and development programmes.

RESULTS

Overall structure of the p52:RelB:DNA complex

The association between the p52 and RelB subunits, through their C-terminal immunoglobulin-like domains, and the overall mode of DNA binding by the two subunits are similar to all other NF- κ B:DNA complexes known so far (Fig 1A; supplementary Fig 1C online). Crystals of the p52:RelB:DNA complex are formed by an unusual DNA-packing arrangement. An asymmetric unit contains three strands of DNA: two as a duplex, and the third strand is base-paired with a symmetry-related DNA strand to form a complementary double-stranded helix (Fig 1B). In this arrangement, the 20-mer-long duplex contains two κ B sites, of which only one is recognized by the heterodimer, whereas the other remains unbound. Therefore, this structure allows us to compare the DNA conformations in their free and protein-bound states. At the other end of the duplex, the 3'-cytosine of the top strand is displaced by the overhanging 5'-cytosine of a neighbouring duplex, resulting in a continuous DNA helix formed by end-to-end stacking and base displacement (Fig 1B).

An overlay of the free and p52:RelB-bound κ B sites shows a striking feature: the backbone of one strand of the bound DNA, but not of the free DNA, has a sharp kink near the centre of the DNA at the G-2/A-1 base step (Fig 1C). Although the DNA base pairing remains intact, the result of this kink is the opening of the bound DNA compared with the unbound DNA. This conformation of the DNA seems to be stabilized by the backbone contacts made by the charged/polar residues of the RelB subunit (discussed later). The overall conformation of the bound DNA seen in this complex has not been observed in other κ B DNAs, including when bound by the p50:RelB heterodimer in which the minor groove undergoes compression on NF- κ B binding.

Protein–DNA interaction

The p52:RelB:DNA complex buries a solvent-accessible surface area of approximately 3,200 Å², which results from several base-specific and nonspecific contacts (Fig 2A). The p52 subunit recognizes a half-site that is made up of 4 bp and an extra helical

cytosine. This extra helical cytosine is in contact with His 62 and Ser 61. In a smooth duplex DNA, if the fifth base pair were a G:C, then that base pair would have been in contact with the histidine as seen in the p52 homodimer–DNA complex (Fig 2B). Therefore, the p52 subunit is destined to recognize a 5 bp 5'-half-site as observed in the p52 homodimer–DNA complex if the correct half-site sequence is present. All other base-specific hydrogen bonding contacts between p52 and DNA are preserved in this complex. Invariant residues Arg 52, Arg 54 and Glu 58 from loop L1 and Lys 221 from the linker are responsible for base-specific interactions (Fig 2B). In addition, Tyr 55 makes van der Waals contacts with the methyl group of T-1 and C⁵ of C-2.

The most striking feature of this complex is that the RelB subunit also recognizes a 5 bp half-site (Fig 2A,C). This is surprising as the terminal G:C base pair is in contact with the histidine residue in p50 and p52 is absent in RelB. The base-specific contact involves *Arg 125* (hereafter, RelB residues are denoted by italics), which accounts for the 5-bp recognition site; corresponding arginines in RelA (Arg 41) and cRel (Arg 29) are not involved in any DNA interactions (Fig 2D). In the p50:RelB complex, Arg 125 is involved in a hydrogen bond with the protein backbone but is not involved in DNA interaction (Fig 2D; supplementary Fig 2 online). The difference observed is due to the conformational change of loop L1. The DNA in the p52:RelB complex occupies the area in which loop L1 of the p50:RelB complex is located. Loop L1 in the complex is rotated away from the DNA surface, resulting in the movement of the *Arg 125* side chain towards the DNA (Fig 2C,D). It is unclear at this stage whether the alternate binding interactions seen in this complex are an inherent property of the complex or the result of the crystal packing described earlier. Overall, the p52:RelB complex seems to interact through the flanking GGG:CCC core elements with very few hydrogen bonding contacts at the central four base pairs.

There are several other distinct features in DNA recognition by RelB. *Tyr 120* makes van der Waals contacts with T + 2. The corresponding tyrosine in other NF- κ B subunits makes sequence-specific van der Waals contacts with two adjacent base pairs. In this complex, a shift in thymine at -1 prevents the contact from occurring. *Lys 274* of RelB is not involved in DNA contact; however, the corresponding residues, Lys 221 of p52, Lys 241 of p50, Arg 187 of RelA and Arg 178 of c-Rel, are involved in forming a contact with a central A:T base pair. A similar observation was made in the p50:RelB:DNA complex structure (Moorthy *et al*, 2007). *Arg 119* makes both base-specific and nonspecific interactions by forming a contact with the DNA backbone and a base pair (Fig 2C). The corresponding arginines in other complexes are involved only in base-specific contacts. Finally, this structure also reveals unusual DNA backbone contacts by RelB; all six central phosphates in one strand of the DNA make direct hydrogen bonds with RelB residues (Fig 2E). Several of these phosphates are in contact with multiple residues; by contrast, p52 residues form only few contacts with the phosphates of the reverse strand. This asymmetric DNA backbone contact might induce the DNA kink mentioned earlier.

Arg 125 of RelB binds to κ B sites selectively

To validate whether distinct contacts made by the RelB subunit in the present structure truly represent the inherent property of the p52:RelB heterodimer or whether they are artificially imposed by

Fig 1 | The structures of the p52:RelB:κB DNA complex and free DNA. (A) Overall structure of the complex. (Left) Ribbon drawing of the entire complex viewed down the DNA helical axis. The RelB and p52 subunits are shown in purple and blue, respectively; the three DNA strands in the asymmetric unit are shown in yellow, green and grey. (Right) View of the complex after rotating 90° along the vertical axis. (B) Schematic representation of the DNA packing as observed in the structure. The DNA duplex shown in grey is unbound. The crystallographic twofold axis and non-crystallographic twofold axis are indicated by an oval and an arrow, respectively. The RelB subunit binds base pairs at positions +1 to +5, whereas the p52 subunit contacts base pairs at positions -1 to -4. The central base pair '0' is at the pseudo twofold axis. The nucleotides out of the continuous DNA helix are written above and below the duplex sequence. The yellow and green colours represent the DNA sequence bound by the p52:RelB heterodimer. The underlined sequences represent symmetry-related DNA. (C) Comparison of the bound (green) and unbound (grey) DNA structures overlaid around the central seven base pairs; the arrow indicates the kinked area.

the crystal contacts, we focused on the functional role of *Arg 125*. We created the *R125A* mutant and compared the binding of the pure recombinant mutant (hereafter, referred to as p52:RelBM) with that of the wild-type heterodimer for κB DNA by using a fluorescence polarization-based binding assay. Our results show that the mutant binds to interferon (IFN)β-κB and ELC-κB sites with a two- to fourfold lower affinity compared with wild type (Fig 3A). By contrast, wild-type and mutant p52:RelB heterodimers bind to the BLC-κB site with similar affinities (Fig 3A). To test whether differential binding has any functional significance in cells, we analysed the luciferase reporter activity. We generated reporters driven by promoters containing IFNβ-κB, ELC-κB, BLC-κB and human immunodeficiency virus (HIV)-κB sites. We found that p52:RelBM is defective in activating transcription from IFNβ-κB and ELC-κB sites, but not from HIV-κB and BLC-κB sites

(Fig 3B). These observations indicate that *Arg 125* is important in recognizing some, but not all, κB sites. To understand the basis for this differential DNA recognition, we examined these κB sequences (Fig 3D). We hypothesize that *Arg 125*-sensitive sequences have more contiguous and centrally located A:T base pairs than do the non-sensitive sites. To determine whether this is true, we examined the transcriptional activity of four more κB sites present in the promoters of MCP-1, E-selectin, cyclin D1 and inducible protein-10 (IP-10) genes. We found that monocyte chemotactic protein-1 (MCP-1), E-selectin and cyclin D1, all of which contain five AT base pairs, show reduced transcriptional activity in cells expressing the mutant heterodimer compared with cells expressing the wild-type heterodimer. By contrast, the IP-10-κB proximal site (IP-10(P)), which contains fewer contiguous AT base pairs, does not show any difference in transcriptional

Fig 2 | Detailed contacts between the protein and DNA. (A) Schematic representation of the DNA contacts made by the p52:RelB heterodimer (p52, blue; RelB, purple). Arrows indicate hydrogen bonds; orange circles indicate van der Waals contacts. (B) Hydrogen bonds (red dotted lines) between the complementary functional groups of R54, R52, H62, S61, E58 and K221 of the p52 subunit and the DNA bases are shown; blue and red colours indicate the basic and acidic groups, respectively. (C) Hydrogen bonds between the functional groups in R117, R119, R125 and E123 of the RelB subunit (purple) and DNA bases and a phosphate are shown as red dotted lines. R125 and K274 are shown in grey. (D) Orientations of R125 of RelB in the current complex are compared with the same in the p50:RelB (Moorthy *et al*, 2007), and with the homologous arginines in RelA and c-Rel in DNA-bound complexes (Huang *et al*, 2001; Chen-Park *et al*, 2002). (E) Hydrogen bonds (dotted lines) between RelB side chains (C122, Y120, R209, K210, Q307, K308, R333 and Q334) and the DNA backbone are shown. Figures are generated using PyMOL.

Fig 3 | RelB Arg 125 of the p52:RelB complex binds to and promotes transcriptional activation from a subset of promoters. (A) Binding isotherms of ELC-κB (left), BLC-κB (middle) and IFNβ-κB (right) by wild-type and mutant p52:RelB complex using fluorescence-based polarization assay. (B) Reporter assay showing the effect of Arg 125 mutation on luciferase activity. All reporter constructs contained 2 × κB sites in the promoter except MCP-1, which contained a single κB site. (C) EMSA assay using ELC-κB, HIV-κB, cyclin D1-κB, IP-10(P)-κB probes and whole-cell lysates of HEK 293T cells co-transfected with full-length p52 and RelB. 'NC' and 'RelB' lanes indicate EMSA using cell extracts prepared from 'empty vector' and 'RelB only' transfected cells, respectively. Please note that RelB does not bind to DNA. Specificity of the p52:RelB:DNA complex is indicated by competition (50-fold excess cold κB DNA) and supershift (α-RelB antibody; sc-226 from Santa Cruz Biotechnology, Santa Cruz, CA, USA) experiments. (D) Sequences of two classes of κB sites: the top three (low A:T) and bottom five (high A:T) sequences are insensitive and sensitive, respectively, to R125 interaction. BLC, B lymphocyte chemokine; ELC, Epstein-Barr virus-induced molecule-1-ligand chemokine; EMSA, electrophoretic mobility shift assay; E Sel, E-selectin; HEK, human embryonic kidney; HIV, human immunodeficiency virus; IFN, interferon; IP-10, inducible protein-10; MCP-1, monocyte chemotactic protein 1; wt, wild type.

activity between wild-type and mutant proteins (Fig 3B,C). As a control, we found that neither p52 nor RelB shows any transcriptional activity on their own, suggesting that reporter activities (or lack of them) observed in p52 and RelB/RelBM co-transfected cells are from the p52:RelB heterodimer (supplementary Fig 3 online). Next, we carried out electrophoretic mobility shift assay (EMSA)

experiments using whole-cell lysates from transfected human embryonic kidney 293T cells. Consistent with our hypothesis, we found that ELC-κB and cyclin D1-κB sites show significant difference between wild-type and mutant heterodimer DNA-binding activity, whereas HIV-κB and IP-10(P)-κB sites do not discriminate between wild-type and mutant heterodimers (Fig 3C).

Fig 4 | Binding affinities of NF- κ B family members to κ B sites. (A) Binding affinities (expressed as equilibrium dissociation constants) are plotted for each κ B DNA and NF- κ B dimers. (B) Sequences of SDF1, IL-2 CD28RE and IL-8 κ B sites. (C) A model of two modes of DNA recognition by the p52:RelB heterodimer. Arg 125 switches its conformation depending on the DNA sequence/conformation. BLC, B lymphocyte chemokine; ELC, Epstein-Barr virus-induced molecule-1-ligand chemokine; HIV, human immunodeficiency virus; IFN, interferon; IL-2, interleukin 2; NF- κ B, nuclear factor- κ B; SDF1, stromal cell-derived factor 1.

Overall, our observations suggest a correlation between binding affinity and transcriptional activity. Thus, these results confirm the idea that DNA sequence dictates RelB-binding mode.

The p52:RelB dimer binds to κ B DNA non-discriminately

On the basis of the differential binding modes of the p52:RelB heterodimer, we reasoned that p52:RelB might be able to recognize a broader spectrum of κ B sites. To test this hypothesis, we measured the binding affinity of the p52:RelB heterodimer for several κ B sites and compared it with that of p50:RelA. We noticed that the p52:RelB and p52:p52 homodimers, for unknown reasons, do not produce strong shifted complexes in EMSA and that the effect is more severe when certain DNA sequences are used. This might explain why it is difficult to observe BLC- κ B and ELC- κ B DNA binding to the p52:RelB dimer by using EMSA. By contrast, solution-based fluorescence polarization assay works well with all NF- κ B proteins, and therefore we have used this assay for the affinity measurement. As shown in Fig 4, p52:RelB

heterodimer binds to all κ B sites tested with reasonably high affinity, with the lowest affinity being 46.5 nM (K_D) for the cyclin D1- κ B site. The p50:RelA heterodimer shows a more variable binding affinity towards these sites. These results indicate that the p52:RelB heterodimer is less discriminatory to diverse κ B sites than the p50:RelA heterodimer. We also suggest that the ability of the p52:RelB heterodimer to form a contact with different DNAs using various strategies allows it to recognize diverse κ B sites efficiently.

DISCUSSION

The X-ray structure of the p52:RelB: κ B DNA complex reveals two marked features: Arg 125 forms a contact with DNA directly, and the heterodimer asymmetrically recognizes the DNA backbone. *In vitro* binding and cell-based reporter assays reveal that Arg 125 in the p52:RelB complex is essential to bind to and drive transcription from some, but not all, κ B sites. Our study has established that κ B sites with a higher number of successive AT

base pairs at or near the centre of the κ B site are susceptible to Arg 125 and thus to protein-induced conformational change (Fig 3C). In this structure, we observe that RelB forms extensive contacts with the central six phosphates on the DNA strand that shows a kink. Therefore, it is likely that the protein-induced conformational change of one DNA strand allows for the distinct contacts formed by Arg 125, Tyr 120 and Arg 119 to DNA, as seen in this structure. We suggest that the protein-induced DNA conformational change is the origin of other recognition features seen in this complex. The DNA used in the current structure contains only four successive AT base pairs, and we infer that natural sequences with a higher number of AT base pairs might undergo structural changes that are even more severe than those seen in this structure. Our structural and biochemical experiments thus suggest that Arg 125 switches its conformation depending on the DNA sequence/conformation it encounters (Fig 4B).

The modular domain architecture of the DNA-binding RHR of NF- κ B subunits endows all NF- κ B dimers with abilities to bind to target sequences by adopting different conformations. When an NF- κ B subunit encounters an incorrect DNA functional group, it can easily avoid forming an energetically unfavourable contact by making global or local conformational change to form alternate contacts. This allows the complex to preserve the overall binding energy. Consistent with this idea, we observed both large and small conformational changes by the RelA subunit when it encountered unfavourable base pairs (Chen *et al*, 1998b, 1999; Chen-Park *et al*, 2002). Results from our binding assay show that the p52:RelB heterodimer is slightly more versatile in DNA recognition than the p50:RelA heterodimer. We suggest that the structural difference in the linker region and perhaps in other areas of RelB makes this subunit even more able to recognize diverse κ B sites by using the DNA backbone-contacting residues as well as the base-contacting residues more than the non-RelB dimers. We are, however, unsure if and how DNA recognition modes of RelB vary when it uses p50 or p52 as a partner. It is important to note here that although the identical κ B DNA was used in both complexes, the two structures look markedly different (Moorthy *et al*, 2007). Further experiments are required to determine whether the observed differences are crystallographic artefacts or represent their solution-binding behaviour.

With the increasing number of NF- κ B-regulated κ B DNA target sites being found, the diversity of these sequences is becoming apparent. Even the stringency of GGG and CC core sequences at the 5'- and 3'-ends is now questionable (Chen *et al*, 1999; Hoffmann *et al*, 2006). Furthermore, there is no κ B sequence known so far that is exclusively targeted and regulated by a specific NF- κ B dimer. One can only say with confidence that some sites might be preferred by a certain dimer over others. Initial reports that most NF- κ B target genes are regulated by the p50:RelA heterodimer were made on the basis of gene expression examined within a few hours of induction. During that period, p50:RelA is the dominant NF- κ B dimer activated owing to its rapid activation by I κ B α degradation (Ghosh & Karin, 2002). The p52:RelB heterodimer is activated over a prolonged period of time and, as suggested earlier, might 'take over' from the p50:RelA heterodimer at later times (Saccani *et al*, 2001). The p52:RelB heterodimer might have an even broader range in gene expression activity than other dimers.

METHODS

Protein expression, purification, crystallization and structure solution. Expression and purification of the heterodimer was carried out as described previously (Fusco *et al*, 2008). Crystallization of the ternary complex, data collection and structure solution have been described in supplementary Fig 1 online and supplementary Table 1 online. The coordinates have been deposited with Protein Data Bank with the accession code 3DO7.

DNA binding and reporter assay. DNA-binding assays by EMSA and fluorescence polarization were carried out as described previously (Phelps *et al*, 2000; Moorthy *et al*, 2007). Luciferase reporter assays have been carried out as follows: cells were transfected with full-length Flag-RelB and/or Flag-p52 expression vectors or empty vector, and the luciferase reporter DNA. All DNA constructs were verified by sequencing. The total amount of plasmid DNA was kept constant for all assays. Transfections were carried out using lipofectamine 2000 reagent following the manufacturer's protocol. Cells were collected 24 h post-transfection and lysed with a buffer containing 20 mM Tris-HCl (pH 7.5), 0.2 M NaCl, 1% Triton X-100, 1 mM EDTA, 2 mM dithiothreitol, 0.1 mM phenyl-methylsulphonyl fluoride and protease inhibitor mixture (Sigma-Aldrich, St Louis, MO, USA). Protein expression was confirmed by Western blot. Luciferase activity assay was carried out using Promega Dual Luciferase Assay kit following the manufacturer's instructions (Promega, Madison, WI, USA).

Supplementary information is available at *EMBO reports* online (<http://www.emboports.org>)

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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