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Structure of an IκBα/NF-κB Complex

Marc D. Jacobs* and Stephen C. Harrison*†‡
*Department of Molecular and Cellular Biology and
†Howard Hughes Medical Institute
Harvard University
Cambridge, Massachusetts 02138

Summary

The inhibitory protein, IκBα, sequesters the transcription factor, NF-κB, as an inactive complex in the cytoplasm. The structure of the IκBα ankyrin repeat domain, bound to a partially truncated NF-κB heterodimer (p50/p65), has been determined by X-ray crystallography at 2.7 Å resolution. It shows a stack of six IκBα ankyrin repeats facing the C-terminal domains of the NF-κB Rel homology regions. Contacts occur in discontinuous patches, suggesting a combinatorial quality for ankyrin repeat specificity. The first two repeats cover an α helically ordered segment containing the p65 nuclear localization signal. The position of the sixth ankyrin repeat shows that full-length IκBα will occlude the NF-κB DNA-binding cleft. The orientation of IκBα in the complex places its N- and C-terminal regions in appropriate locations for their known regulatory functions.

Introduction

The transcription factor NF-κB, present in many cell types, mediates activation of the immune and inflammatory responses (reviewed in Baeuerle and Henkel, 1994; Ghosh et al., 1998). NF-κB is a dimer of proteins belonging to the Rel family, including p65 (RelA), p50, p52, and c-Rel, all of which have a 300-residue "Rel homology region" (RHR). The structures of the RHRs of p50 and p65 bound to DNA show that each consists of two domains with immunoglobulin-like folds (Ghosh et al., 1995; Müller et al., 1995; Chen et al., 1998a, 1998b). The C-terminal domains (RHR-c) are responsible for dimerization with other Rel family proteins, and the different homo- and heterodimers thus formed have somewhat varied DNA binding specificities. Both domains of the RHR contact DNA, but sequence-specific interactions come primarily from loops in the N-terminal domain (RHR-n).

Association with inhibitory proteins of the IκB family, in particular those such as IκBα, IκBβ, and IκBϵ, retains NF-κB in the cytoplasm. The precursors of p50 and p52, known as p105 and p100, respectively, contain IκB-like regions near the C termini of their polypeptide chains. When one of these precursors is a partner in a heterodimer (e.g., p105:p65), the complex also remains in the cytoplasm (Henkel et al., 1992; Rice et al., 1992). All the IκB proteins appear to inhibit NF-κB activity by masking a nuclear localization signal (NLS), located just at the C-terminal end of the RHR in each of the NF-κB subunits (Beg et al., 1992; Ganchi et al., 1992; Zabel et al., 1993). In addition, IκB association interferes directly with the capacity of NF-κB to bind DNA (Baeuerle and Baltimore, 1988; Zabel and Baeuerle, 1990). Indeed, one member of the IκB family, Bcl-3, is found primarily in the nucleus and appears to function mainly through its effects on DNA binding (Ghosh et al., 1998).

Each of the IκB proteins contains six or more 33-residue-long ankyrin repeats, named for their homology to repeats in the erythrocyte protein ankyrin (reviewed in Bork, 1993). Ankyrin, which has 24 such repeats, links spectrin, a major erythrocyte cytoskeletal component, to the band-3 protein of the red blood cell membrane (Bennett and Branton, 1977; Bennett and Stenbuck, 1979; Lux et al., 1990). Ankyrin repeats are found in diverse proteins, including factors regulating the cell cycle in yeast and development in *Drosophila*. They serve as interaction elements for specific protein associations.

The best-characterized IκB protein is IκBα, composed of three regions: an N-terminal region, which regulates signal-dependent degradation; an ankyrin repeat domain; and a C-terminal PEST region regulating basal degradation (Davis et al., 1991; Haskill et al., 1991). In response to stimuli such as cytokines or products of bacterial/viral infections, IκBα is phosphorylated by specific kinases at two sites near the N terminus (Ser-32 and Ser-36) (DiDonato et al., 1996, 1997; Lee et al., 1997, 1998). The phosphorylated protein is then ubiquitinated at Lys-21 and Lys-22, leading to proteosome-mediated degradation (Palombella et al., 1994; Traenckner et al., 1994; Chen et al., 1995; Scherer et al., 1995; Rodriguez et al., 1996; Roff et al., 1996). Removal of IκB unmasks the NLS of NF-κB and allows translocation to the nucleus. When associated with NF-κB, IκBα also binds and inhibits the catalytic domain of protein kinase A (PKAc). Degradation of IκBα relieves this inhibition, leading to phosphorylation of p65 (at Ser-276) and increasing its strength as a transcriptional activator (Zhong et al., 1997, 1998). Thus, IκBα is a multifunctional inhibitor of NF-κB, blocking nuclear translocation, DNA binding, and phosphorylation by PKA.

We describe here the structure of the ankyrin domain of IκBα bound to an NF-κB heterodimer containing the RHR of p65 and the RHR-c of p50. The six ankyrin repeats form a curved stack, which faces the dimerization domains of NF-κB. The NLS of p65, not ordered in NF-κB bound to DNA, is sequestered by the first two ankyrin repeats as an α-helical segment. A similar NLS is an extended chain when complexed with karyopherin-α (Conti et al., 1998). Thus, the conformation of such a signal depends upon the context in which it is recognized. The interface between IκBα and NF-κB consists of several patches, each involving a relatively small number of residues. The pattern of conserved residues in other IκB proteins indicates that they would bind to Rel dimers in a similar manner and with the same register and orientation. Many of the residues most conserved

‡To whom correspondence should be addressed (e-mail: harrison@crystal.harvard.edu).

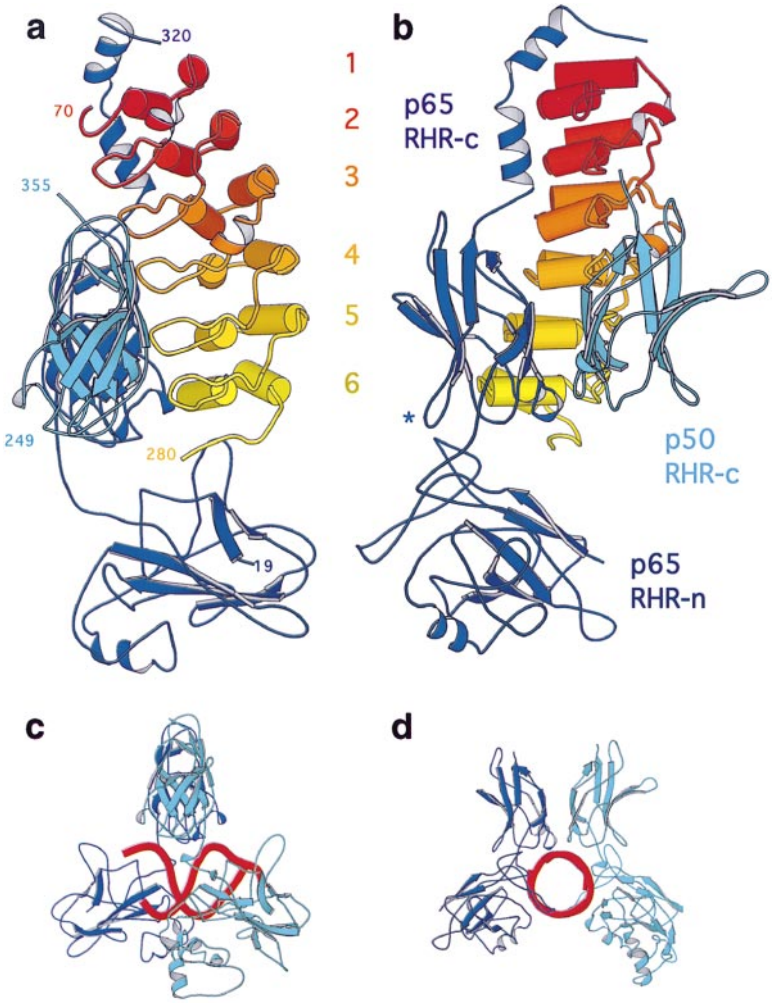

Figure 1. The Structure of the NF-κB/IκBα Complex, with DNA Bound NF-κB as a Reference

(a) A ribbon drawing of the ankyrin domain of IκBα bound to NF-κB. The RHR-c and -n domains of p65 are shown in dark blue, the RHR-c domain of p50 in light blue, and the IκBα ankyrin domains shaded from red at the N terminus to yellow at the C terminus. The RHR-n domain of p50 is not present in this structure. Each ankyrin repeat is numbered in its corresponding color, and the α helices of IκBα are represented as cylinders. Residue numbers of the termini of the model are shown in the left panel.

(b) View of NF-κB/IκBα complex rotated 90° from the view in (a). The asterisk indicates the p65 phosphorylation site, Ser-276.

(c and d) NF-κB bound to DNA (Chen et al., 1998a). These views correspond to the orientations of NF-κB in (a) and (b), respectively, and use the same colors. The DNA is shown in red as a ribbon through backbone phosphate positions. Figures made using the program MOLSCRIPT (Kraulis, 1991).

among ankyrin repeats are internal and define the interactions within a repeat and between adjacent repeats. The regular structure of the ankyrin stacks, with residues responsible for interprotein contacts clustered in groups on the surface, suggests that ankyrin repeats have evolved as scaffolds to display a set of oriented contact residues for protein recognition.

Results and Discussion

Formation of Stable NF-κB/IκBα Complexes

When NF-κB species containing the RHR of p50 (residues 40–366) and/or the RHR of p65 (residues 1–305) were combined with various IκBα fragments, complexes could be detected by nondenaturing gel electrophoresis, but they were not stable on the time scale needed for gel filtration (~2 hr), and no crystals were obtained. Complexes containing a longer p65 species (residues 1–323), which includes 19 residues after NLS, could be purified by gel filtration. Titration of IκBα against NF-κB indicated that the stoichiometry was 1 IκBα:1 NF-κB dimer, consistent with other studies (Ernst et al., 1995). Complexes could also be detected with homodimers of the C-terminal domain of the p50 RHR (residues 243–366). We therefore screened for crystallization of complexes containing p65 (1–323) and p50 RHR-c, with various IκBα fragments guided by proteolysis studies (Jaffray et al., 1995). Those with IκBα (69–288) yielded good crystals. The structure was determined by using a combination of molecular replacement and multiple isomorphous replacement. The two complexes in the asymmetric unit are essentially identical, except for small differences in the first ankyrin repeat and the five C-terminal residues of p65, where a crystal contact is made. The final model contains residues 71–280 of IκBα, 249–355 of p50, and 19–320 of p65.

Overview of the Structure

The IκBα fragment in this complex includes the six ankyrin repeats and 13 residues C-terminal to the last repeat, of which 5 are ordered (Figure 1). The structures of several proteins containing ankyrin repeats have shown that they form stacked layers with an overall left-handed twist (Gorina and Pavletich, 1996; Luh et al., 1997; Batchelor et al., 1998; Brotherton et al., 1998; Russo et al., 1998; Venkataramani et al., 1998) (Figure 2). Each repeat consists of a β loop and two antiparallel α helices. The loop is roughly perpendicular to the axis

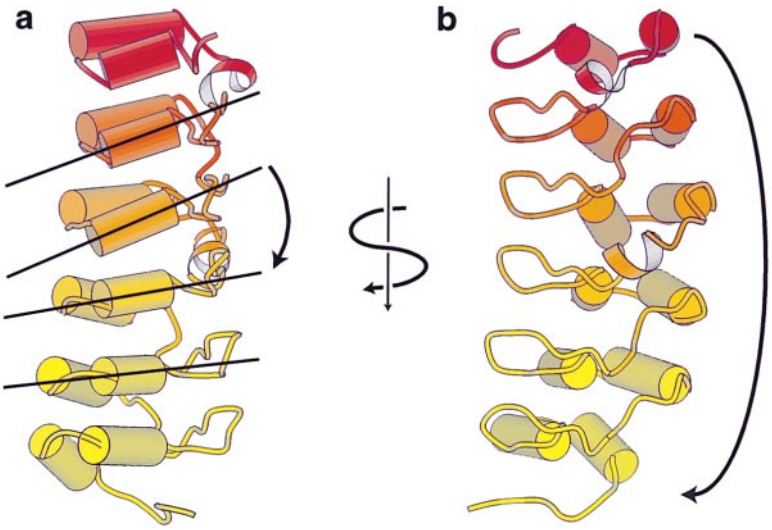

Figure 2. Curvature and Twist of the IκBα Ankyrin Repeat Stack

(a) View showing how curvature in one direction is introduced by "kinks" due to α-helical inserts between repeats.

(b) View orthogonal to the one in (a), showing how curvature in the other direction correlates with different vertical spacings between inner and outer helices in the stack. The overall twist is shown by the helical arrow in the center. Figures made using the program MOLSCRIPT (Kraulis, 1991).

of the helices, giving the stack an L-shaped cross section. The image of a hand, with fingers held at right angles to palm, is useful: the loops are the fingers, and the palm is the stack of helical hairpins. The "inner" and "outer" helices of each hairpin face the concave and convex sides of the cupped hand, respectively. The six repeats of IκBα lie with the concave side of the stack facing the NF-κB dimerization module and its NLS extension (Figures 1 and 2). Each of the ankyrin repeats stacks on the previous one at a small angle, causing the stack to bend toward the face of the inner helix, and thus toward NF-κB. Repeats 1 and 2 of IκB contact the p65 NLS and residues just C-terminal to it, which extend away from the RHR; repeats 4–6 contact the RHR-c domains of p50 and p65 (Figure 1). The structures of the domains in the dimerization module and of p65 RHR-n are essentially identical to those in the DNA-bound forms of NF-κB (Ghosh et al., 1995; Müller et al., 1995; Chen et al., 1998a, 1998b). Lack of extensive contacts between the fragment of IκBα in this complex and the p65 RHR-n and the paucity of interactions between the p65 RHR-c and -n domains allow p65 RHR-n to rotate, relative to its orientation in the DNA complex, by about 180° (compare Figures 1a and 1c). The position of the p65 RHR-n domain is partly determined by crystal packing.

IκBα Ankyrin Repeats

The internal structures of the ankyrin repeats are very similar to each other, and their sequences are closely related (Figure 3). The tip of the loop at the start of each repeat is a bulged β turn, stabilized by hydrogen bonds between main-chain groups of the residue at position 5 and those at 8 and 9 and between the side chain at 5 and the amide at 7. In the fifth repeat, where there is a proline at 5, a glutamate at 4 makes similar interactions. The turns of successive repeats are linked by a row of main-chain hydrogen bonds between positions 7 of one and 6 of the next. The interactions of the helices determine the overall architecture of the domain. The inner and outer helices in each repeat diverge by about 20° from the tight turn that joins them. Both make hydrophobic contacts with their counterparts in adjacent repeats "above" and "below." The outer helices generally have larger hydrophobic side chains in these interfaces, producing curvature in the stack (Batchelor et al., 1998) (Figure 2b). The C-terminal end of the inner helix packs between the two helices of the succeeding repeat, generating the left-handed twist. The inner helix contains the characteristic IκB ankyrin repeat motif, TPLHAV (positions 10 to 16). The hydroxyl of the threonine (or serine) donates a hydrogen bond to the histidine, which in turn fixes the position of the loop in the next repeat by donating hydrogen bonds to the carbonyls of positions 3 and/or 9. The solvent-exposed residues at the "bottom" surface of repeat 6 are largely polar, but the "top" of repeat 1 is hydrophobic.

Alignment of the sequences of known mammalian IκB family members (Figure 3) shows that the greatest similarity occurs among repeats at the same position in different IκB proteins (e.g., repeat 3 of IκBα and IκBβ) rather than among different repeats in the same protein. This pattern of similarity implies that a particular repeat has a related function in each of the IκB family members. We note also a regularity in the inserts between adjacent ankyrin repeats: there are two to six residues following repeat 1, a larger insert after repeat 3, and a single residue after repeat 4. These interrepeat sequences are not part of the contact surface with NF-κB, and the long inserts between repeats 3 and 4 in IκBβ and IκBε could readily loop out of the stack. The short helical segments in IκBα after repeats 1 and 3 introduce local kinks, creating the crescent shaped profile seen in the view of Figure 2a; the corresponding inserts in the other IκB proteins are likely to do the same. The sixth repeat of IκBα acts as a cap on the stack of ankyrin repeats, and its sequence is the most divergent. The sixth repeat of IκBβ probably has a similar role. Both lack the histidine at position 13 that interacts with the loop of the next repeat. The ankyrin domains of p100, p105, and Bcl-3 have seven repeats; the sequence of the seventh repeat is the least conserved (and lacks the histidine). The alignment in Figure 3 shows that IκBε is also likely to have

Figure 3. Sequence Alignment of IκB and Rel-Homology Proteins Showing Contacts in the Complex

(a) The sequences of the ankyrin repeat domains of IκB proteins are aligned in seven sets, one set for each ankyrin repeat. The numbers on the left refer to the residue number of IκBα, and the numbers on the top indicate the position within the ankyrin repeat. Secondary structural elements are shown at the top with the β turn at the left followed by two α helices. Residues between adjacent repeats are listed in the rightmost columns; where there are many residues between repeats, the number of such residues is shown in parentheses. Residues of IκBα that contact p65 and p50 are boxed in blue and red, respectively. Residues that are the same in three or more sequences at the same position are shaded gray (valine, leucine, and isoleucine are considered identical for this figure).

(b) Sequences of p65, p50, and c-Rel are aligned. Residues interacting with IκBα are enclosed in boxes colored according to the repeat contacted: 1, red; 2, green; 3, light blue; 4, dark blue; 5, gold; 6, black. The secondary structure of p50 and p65 is indicated at the top of the alignment with β strands as arrows and α helices as rectangles. The position of the NLS is indicated in helix 1.

seven ankyrin repeats and that structurally it falls in a class with p100, p105, and Bcl-3.

Interaction of IκB and NF-κB

There are three regions of contact between NF-κB and IκBα in the complex we have crystallized. The contacts do not create a single extended buried surface but rather several smaller patches (Figure 4). Various parts of repeats 1 and 2 contact the C-terminal extension of p65, including its NLS; the tips of the loops ("fingers") in ankyrin repeats 4–6 contact p50 along the interface of the paired dimerization domains; and the inner helices of repeats 5 and 6 contact the dimerization domain of p65. Repeat 3 has relatively little contact with NF-κB. In the avian IκB protein, pp40, mutations in the inner helix of the third ankyrin repeat did not disrupt NF-κB binding, whereas mutations in the other repeats prevented complex formation (Inoue et al., 1992).

IκBα imposes an α-helical conformation on the segment containing the p65 NLS, which is disordered in structures of NF-κB bound to DNA as well as in structures of free p50 or p65 RHR-c domains (Ghosh et al., 1995; Müller et al., 1995; Huang et al., 1997). Residues 291–314 of p65 form two α helices, joined at 305. The NLS, residues 301–304, has the sequence Lys-Arg-Lys-Arg. The side chains of three of these residues interact with IκBα through salt bridges to acidic groups (Figure 5a). A stack of ankyrin modules has a periodicity of about 10 Å, corresponding to the packing diameter of the α helices at the core of each repeat. An α-helical polypeptide likewise has roughly a 10 Å periodicity along its axis (seven residues in approximately two turns). The match between these two distances allows the isoleucines at position 17 in repeats 1 and 2 to make essentially identical contacts with main-chain atoms of p65, two α-helical turns apart. In one of the two complexes in the crystallographic asymmetric unit, residues 315–320 of p65 form an extended strand, which "caps" the first ankyrin repeat. As noted earlier, residues 307–323 contribute significantly to the observed lifetime of the complex in solution, and we ascribe this effect to a set of hydrophobic interactions (see Figure 5a, caption).

The p50/ IκBα contact is a stripe along p50 strands *a*, *a′*, and *g* (see Figure 3b). There are several side-chain and main-chain hydrogen bonds between residues in strands *a* and *a′* and the β turn "fingertips" of repeats 5 and 6. A hydrophobic cluster opposite Tyr-181 in the fingertip of repeat 4 includes side chains of several p50 residues. Other IκB proteins have an aromatic residue at the position corresponding to 181, but not all Rel family members have the complete hydrophobic patch that faces it. The p65/IκBα contact is largely between the *c′d* loop (residues 238–243) and the inner helix of repeat 6.

An extended network of salt bridges and hydrogen

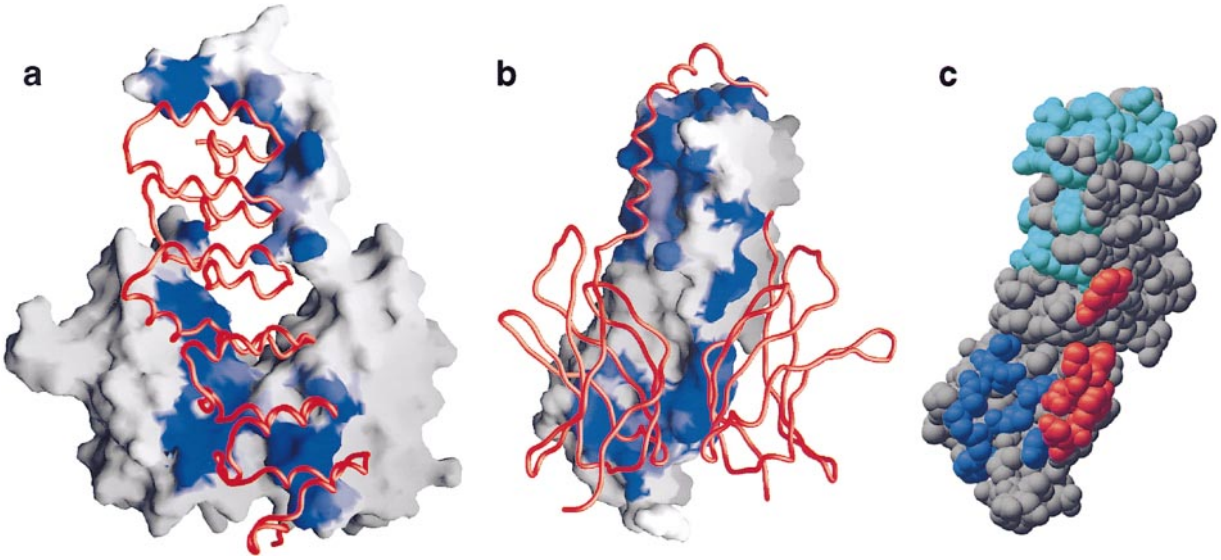

Figure 4. Contacts between NF-κB and IκBα

(a) The backbone of IκBα is shown as an orange strand against the van der Waals contact surface of NF-κB. The surface is colored dark blue where the center of an atom of IκBα is within 3.8 Å of an atom of NF-κB, and graded from light blue to white for distances up to 4.5 Å. This figure is related by a 180° rotation relative to the view in Figure 1b. The thin strip of continuity between the p50 contact (left) and the p65 contact (right) is the conserved salt-bridge network shown in Figure 5b.

(b) The backbones of the dimerization domains of NF-κB are shown as orange strands against the van der Waals contact surface of IκBα. This view is in the same orientation as Figure 1b. Note how the fingers of IκBα project forward toward the edge of the p50 RHR-c.

(c) Residues of IκBα where side chains make contacts with NF-κB are shown in color on a space-filling representation of the ankyrin repeat stack. The orientation of the model is the same as in Figures 1b and 4b. Residues contacting the p50 RHR-c are in red; those contacting p65 RHR-c are in dark blue; those contacting the p65 NLS and the segment C-terminal to it are in light blue. These residues are also indicated by colored boxes in Figure 3a. (a) and (b) were made using the program GRASP (Nicholls et al., 1991), and (c), using the program RIBBONS (Carson and Bugg, 1986).

bonds provides the only continuity between the IκBα interface with p50 and its contact with p65 (Figures 4a and 5b). The residues that participate are largely conserved among the various IκB and Rel family members.

Register, Symmetry, and Selectivity

What can we say, from the contacts in this structure, about other IκB/Rel protein interactions? Conservation of many contacting residues within both families (Figure 3) requires that the register of ankyrin repeats and Rel-protein elements be the same as in the complex we have studied. The salt-bridge network illustrated in Figure 5b is particularly convincing in this regard. Therefore, repeats 1 and 2 of all IκB proteins will participate in the masking of an NLS, and the C termini of their ankyrin stacks will project toward the DNA-binding regions of their NF-κB partners. In p105 and p100, a 25-residue glycine-rich segment connects the RHR at the N terminus and the IκB-like region at the C terminus. Assuming that the ankyrin domain of p105 and p100 interact within p65/p105 or p65/p100 heterodimers as in our structure, the glycine-rich stretch can extend outward as a simple loop between the two parts of the precursor.

IκBα can associate with homodimers of p50 or of p65 as well as with the heterodimer (Ghosh et al., 1998). The structures of the p50 and p65 RHR-c domains are extremely similar, and the residues contacting IκB are largely conserved (Figure 3b). Thus, the contacts of IκBα with p50 described above will be reasonably stable when made with the corresponding residues in p65, and those described with p65 will be stable when made with p50. Moreover, the sequence of residues in the vicinity of the p50 NLS suggests that this region could form a structure that caps the IκBα ankyrin stack just as does the extension of p65 in the present complex. In the crystal structure, we only see one orientation of IκB on the heterodimer—the fingers contact p50 and the inner helices contact p65, and not the other way around. Modest differences among contacting residues at homologous positions on both sides of the IκB/NF-κB interface—for example, the patch covering Tyr-181 of IκBα—could readily establish a strong preference for one orientation over the other.

If both faces of NF-κB can interact with IκB, why are there not two IκB molecules in the complex? Simultaneous binding of two IκBα's would require significant rearrangement of the structure seen here. Rotation of this model, swapping p50 for p65 and optimizing the overlap of the dimerization domains of the two NF-κB components, shows that were two IκB molecules present, the NLS of one NF-κB subunit would collide with repeats 1 and 2 of the IκB facing the other subunit. Ordering of the NLS is thus important for the asymmetry of the complex.

Despite binding promiscuity when overproduced, different IκB's are thought to be specific inhibitors of particular sets of NF-κB's (Ghosh et al., 1998). Where contacting residues are not conserved, in either partner in the complex, we can see no correlation between sequence variation and apparent functional target. For

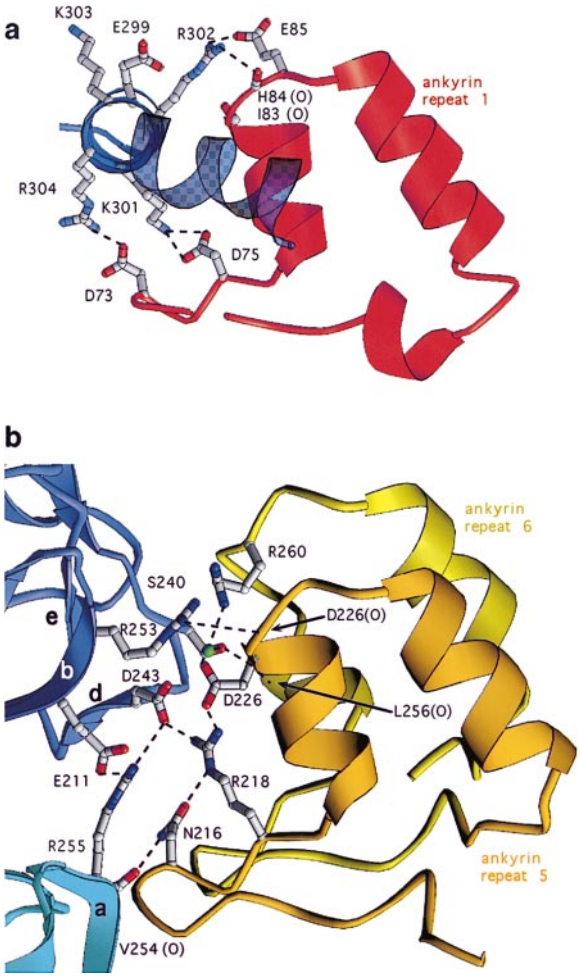

Figure 5. Details of the Interactions between NF-κB and IκBα

(a) Interactions between the p65 nuclear localization sequence of p65 and IκBα. The first ankyrin repeat is depicted in red. The first C-terminal helix of p65 is perpendicular to the page and is drawn in blue, while the second helix is drawn as a transparent blue ribbon. Contacts are drawn as dashed lines. This view corresponds to the model in Figure 1a as seen from above, looking down through the stack of ankyrin repeats. Lys-301 has a salt bridge to Asp-75; the side chain of Arg-302 has a salt bridge to Glu-85 and caps the inner helix of repeat 1 by donating hydrogen bonds to main-chain carbonyls of residues 83 and 84; and Arg-304 has a salt bridge to Asp-73. Not shown in the figure is a set of hydrophobic interactions among Phe-309, Ile-312, Met-313, and Phe-318 in p65 and Phe-77, Leu-80, Ala-81, Leu-89, and Val-93 in IκBα, and a hydrogen bond from Thr-305 to His-85.

(b) Polar interactions at the interface between IκBα, p50, and p65. The fifth and sixth ankyrin repeats are drawn in orange and yellow; p65 is shown in dark blue and p50 in light blue, both with strand labels from Figure 3b. Hydrogen bonds to backbone carbonyls are labeled as (O). The green sphere is a tightly bound water molecule. Figures made with the program RIBBONS (Carson and Bugg, 1986).

example, there are no positions within the six ankyrin repeats where Bcl-3, p105, and p100 (all thought to target homodimers of p50 or p52) have one residue, and IκBα and IκBβ (both thought to target heterodimers) have another. Specific partnering may be as much a consequence of coexpression and other regulatory phenomena as it is of selective binding. There may also be contributions from parts of IκB not included in our structure (for example, a possible interaction of part of the N-terminal region of IκBα with the NLS of p50, discussed below).

Functions of IκBα

Known IκBα activities include cytoplasmic retention of NF-κB, inhibition of DNA binding by NF-κB, recruitment and inhibition of PKA in the IκB/NF-κB complex, and clearance of NF-κB from the nucleus. Other IκB proteins appear to share at least some of these properties (Ghosh et al., 1998).

Cytoplasmic retention requires masking of both nuclear localization sequences in an NF-κB heterodimer (and presumably both sequences in a homodimer as well). Sequestration of the p65 NLS is evident in the structure of the IκBα/NF-κB complex (Figures 1b and 5a). The number of residues between the end of the ordered dimerization domain and the core NLS sequence is the same in other NF-κB subunits, and the character of surrounding residues that contact IκB is also conserved (Figure 3b). Moreover, the three residues of the p65 NLS that have salt bridges to acidic residues in IκBα are lysines or arginines in all other NF-κB subunits. Thus, IκBα is likely to mask one of the two NLSs in any NF-κB family member dimer with the same structure and interactions as seen here. The p50 NLS is disordered in our crystal. Deletion and mutation experiments show that residues between 44 and 52 of IκBα, not present in the crystalline fragment, are necessary for cytoplasmic retention of p50 homodimers (Latimer et al., 1998). This part of IκBα could easily fold back to mask the NLS of p50 (Figure 6), either by binding the NLS directly or by indirectly preventing interactions with the nuclear import machinery.

Inhibition of DNA binding requires sequences C-terminal to the ankyrin domain. A detailed study using nested deletions has suggested that the particular IκB truncation present in our crystals is compatible with DNA binding but that the presence of even a few more residues at the C terminus leads to inhibition (Luque and Gélinas, 1998). The structure indeed shows that the C terminus of the IκB fragment approaches the DNA-binding cleft and that any extension would probably overlap with bound DNA. A complete C-terminal segment of IκB might interact directly with the N-terminal domain of the RHR (Kumar et al., 1992) and might also constrain its orientation. The seventh ankyrin repeat, present in p100, p105, Bcl-3, and IκBε (Figure 3a), is likely to block the DNA cleft. We believe that all of these forms of IκB should interfere with DNA binding. Depending on its phosphorylation state, Bcl-3 has been observed either to inhibit DNA binding by p50 homodimers or to form ternary complexes (Bundy and McKeithan, 1997). The latter will probably require a conformational change in the C-terminal region of Bcl-3.

The N- and C-terminal regions of IκBα, although not present in our structure, have locations consistent with their known or likely functions. The residues of IκBα that determine its signal-dependent proteosomal degradation are all in the N-terminal region. As illustrated in Figure 6, we expect them to be unstructured and fully

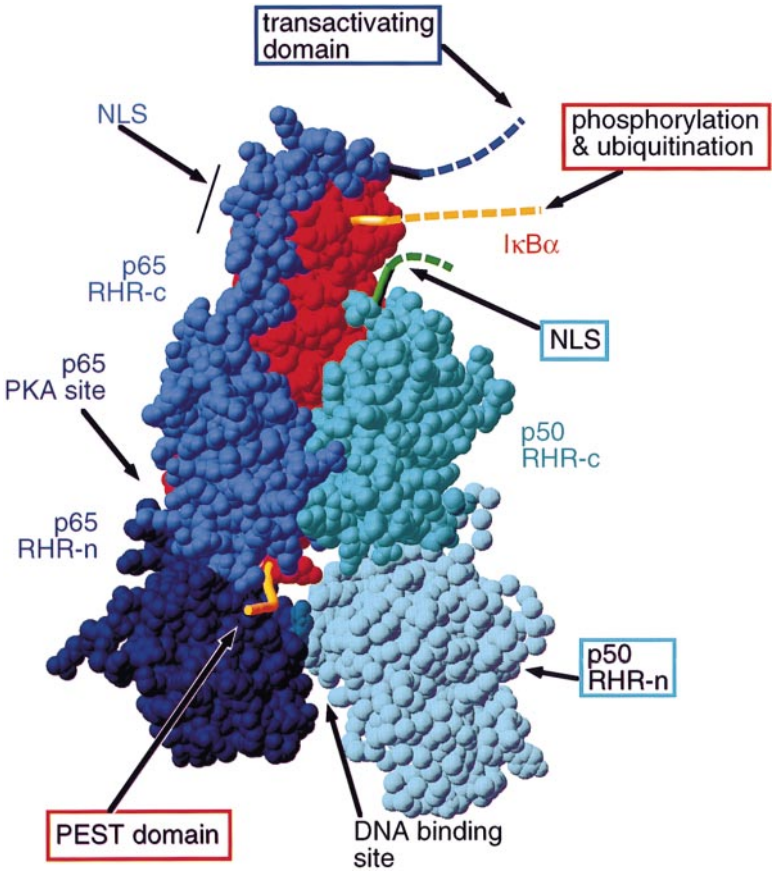

Figure 6. Diagram of the IκB/NF-κB Complex, Showing Additional Interactions and Relative Positions of Domains Not Included in the Crystal Structure

Space-filling representations of the ankyrin domain of IκBα and of the RHRs of p65 and p50 are shown, with strands to represent N- or C-terminal extensions. The C-terminal extension of p65 includes segments likely to interact with the transcription machinery. The C terminus of p50 following the NLS is a glycine-rich tail. In p105, this tail connects to an IκB domain. The figure shows that this connection can be a simple loop. The N-terminal 69 residues of IκBα include the sites for phosphorylation and ubiquitination as well as residues thought to mask the p50 NLS. The C-terminal extension of IκBα occludes the DNA-binding site; it contains a proline, glutamic-acid, serine, and threonine-rich segment (PEST domain), which regulates basal turnover (Verma et al., 1995). The color scheme conforms to Figure 1. Features with labels in colored boxes are parts of the complex not present or not ordered in the crystal structure. The p50 RHR-n has been modeled in an orientation, relative to p50 RHR-c, similar to the orientation of p65 RHR-n relative to p65 RHR-c in the structure. Both of the RHR N-terminal domains may actually adopt a variety of orientations, due to the flexibility of the RHR linker. Figures made with the program RIBBONS (Carson and Bugg, 1986).

accessible, both for modification by a kinase and for ubiquitination. It has been suggested that the residues around position 272 in IκB serve as a nuclear export signal, allowing clearance of nuclear IκB/NF-κB complexes (Arenzana-Seisdedos et al., 1997). This segment of IκBα lies just at the C terminus of our fragment, and it is indeed exposed in the structure. The locus of interaction between PKA and IκBα has not yet been determined. The orientation of the ankyrin stack in the complex places the C-terminal region of IκB in the vicinity of the PKA phosphorylation site at the "bottom" margin of the p65 dimerization domain. If the recruitment sequences for PKA are in the C-terminal part of IκBα, then PKA in the complex will lie near its enzymatic target site (Figures 1b and 6).

Ankyrin Repeats as Modular Recognition Units

The most conserved ankyrin repeat residues determine the structure of a repeat and the stacking interactions with its neighbors (Gorina and Pavletich, 1996; Luh et al., 1997; Batchelor et al., 1998; Venkataramani et al., 1998). The overall curvatures and twist of the stack can vary both by changes in these residues and by insertions between repeats as described earlier, and this limited variation in the geometry of the array can partially fit it to the surface of a target protein. Nonetheless, residues displayed on the ankyrin stack appear to be relatively independent of each other (Figure 4c). Because the surfaces of IκB and NF-κB are only roughly molded to each other, the contacts segregate into discrete patches rather than merging into a continuous interface. Changes in one patch need not be correlated with changes in another, and the two can evolve independently. Moreover, the linear repeat of the stack has probably allowed evolution to work with a large number of combinations of displayed residues.

A conserved scaffold that presents independently variable residues can allow recognition of diverse protein surfaces. In the recently published structures of cyclin-dependent kinase (Cdk) inhibitors complexed with Cdk6, four ankyrin repeats in the inhibitor molecules bind the small lobe of the kinase (Brotherton et al., 1998; Russo et al., 1998). The concave surface of the ankyrin stack faces Cdk6, and the residues that participate in the contacts are in similar positions to those that form the interface in our structure. These residues are primarily in the β loop "fingers" and near the base of the inner helix. In two other ankyrin domain complexes, the repeats use largely their fingertips for interaction (Gorina and Pavletich, 1996; Batchelor et al., 1998). We suggest an analogy with antibody recognition, in which conserved framework elements present a set of variable loops.

The p65 NLS is a "classical" nuclear import motif, now known to be recognized by karyopherin-α and its homologs (Adams and Adams, 1994; Görlich et al., 1994). Its α-helical structure seen here is very different from the extended-chain conformation adopted by the closely related SV40 NLS when bound to karyopherin-α (Conti et al., 1998). In both cases, it is presumably the

Table 1. Data Collection, Phase Determination, and Refinement Statistics

Data Set	Native A	Native B	$K_3UO_2F_5$ A	$K_3UO_2F_5$ B	$UO_2(CH_3COO)_2$
Resolution (Å)	20–3.3	20–2.7	20–3.3	20–3.3	20–3.3
Completeness (%)[a]	98 (89)	92 (83)	99 (97)	93 (84)	98 (94)
R_{sym} (%)[a]	7.6 (10.8)	5.0 (20.6)	6.0 (9.3)	5.7 (7.7)	6.7 (9.3)
Unique reflections (redundancy)	25089 (3.0)	41990 (3.7)	25390 (3.6)	23840 (4.3)	25075 (3.2)
Phase Determination (Using Native A)					
R_{deriv} (sites)			0.32 (7)	0.29 (7)	0.31 (5)
R_{cullis}			0.76	0.82	0.78
Phasing power			1.12	0.89	0.95
Mean figure of merit		0.46			
Refinement (Using Native B)	20.0–2.7 Å all data		9809 atoms		
R factor (%)[a]	22.2 (31.5)		39742 reflections		
Free R factor (%)[a]	26.5 (37.4)		2093 reflections		
Rms bond length (Å)	0.010				
Rms bond angles (°)	1.7				

Native A and Native B refer to data collected from slightly nonisomorphous crystals. $K_3UO_2F_5$A and $K_3UO_2F_5$B refer to derivatives with sites in common and varied occupancies.
$R_{sym} = \Sigma|I - <I>|/\Sigma I$, where I is the observed intensity, $<I>$ is the mean intensity of multiple observations of symmetry-related reflections.
$R_{deriv} = \Sigma||F_{PH}| - |F_P||/\Sigma|F_P|$, where $|F_P|$ is the protein structure factor amplitude and $|F_{PH}|$ is the heavy-atom derivative structure factor amplitude.
$R_{cullis} = \Sigma||F_{PH}| - |F_P + F_H||/\Sigma|F_{PH} - F_P|$, where F_H is the heavy-atom structure factor amplitude.
Phasing power $= <|F_H|>/<||F_{PH}| - |F_P + F_H||>$; $||F_{PH}| - |F_P + F_H||$ is also known as the residual lack of closure error.
Mean figure of merit $= <\Sigma P(\alpha)\, e^{i\alpha}/\Sigma P(\alpha)>$, where α is the phase and $P(\alpha)$ is the phase probability distribution.
$R = \Sigma||F_O| - |F_C||/\Sigma|F_O|$, where $|F_O|$ and $|F_C|$ are observed and calculated structure factor amplitudes, respectively. R_{free} is calculated for a randomly chosen 5% of reflections not used for refinement.
[a] Values for the highest resolution shell are listed in parentheses.

associating protein (IκBα; karyopherin-α) that imposes a particular conformation on the NLS peptide. We have noted the suitability of the ankyrin repeat stack for regular contact with an α helix. We expect that the p65 NLS will be an extended chain, not an α helix, when bound to karyopherin-α. Signals for subcellular localization are in general short peptide segments, recognized by their sequence and in some cases by their consequent conformational propensities, but rarely as prefolded surfaces. Many other interactions in transcriptional regulatory complexes are likely to have this "peptide-recognition" character (Harrison, 1996).

A related structure is reported in another paper in this issue of *Cell* (Huxford et al., 1998).

Experimental Procedures

Crystallization and Data Collection

IκBα proteins were expressed in *E. coli* and purified as fusions with glutathione S–transferase (Jaffray et al., 1995). p50 RHR-c (residues 243–366) was purified by a procedure similar to that used for the RHR of p50 (Sodeoka et al., 1993a). The p65 RHR (residues 1–323) was expressed in *E. coli* using a T7 polymerase expression plasmid, pLM1 (Sodeoka et al., 1993b), and purified to homogeneity through ion-exchange and gel-filtration column chromatography (R. Chopra, unpublished data). NF-κB heterodimers were formed by incubating an excess of p50 RHR-c with p65 at 37°C for 1 hr. IκBα was added, and the resulting complex was purified by gel filtration chromatography. Crystals were grown in hanging drops at 4°C containing 2 μl protein stock solution (20 mg/ml protein, 75 mM ammonium acetate, 10 mM dithiothreitol, 1 mM ethylenediamine tetraacetic acid, 0.05% n-octyl-β-D-glucopyranoside, 8 mM Tris [pH 8.0]) mixed with 1 μl well solution (25 mM sodium acetate [pH 5.0], 8%–10% polyethylene glycol (PEG) 6000. Crystals in space group $P2_1$ ($a = 49.1$, $b = 91.1$, $c = 191.0$ Å, $\beta = 96.3°$, with two complexes per asymmetric unit) grew in about 10 days to a size of 200 × 200 × 25 μm.

Before data collection, crystals were equilibrated in 12% PEG 6000 and 35% (w/v) glycerol and flash frozen in liquid nitrogen. Native and derivative data (Table 1) were recorded at the X25 beamline of the National Synchrotron Lightsource at Brookhaven National Laboratory using the Brandeis 4-element CCD detector and a Mar345 image plate detector. Intensities were integrated and scaled using the programs DENZO and SCALEPACK (Otwinowski, 1993).

Structure Determination

Partial phase information was obtained from molecular replacement (MR) with the program AMORE (CCP4, 1994) using the dimerization domains from the p50 homodimer or the p50/p65 heterodimer structures (Müller et al., 1995; Chen et al., 1998a) as search models. These phases were used to confirm initial heavy atom positions and to locate remaining sites. Heavy atom parameters were refined using MLPHARE (CCP4, 1994). The program SIGMAA (CCP4, 1994) was used to combine heavy atom and molecular replacement phases using native data set A. The initial map was improved by noncrystallographic symmetry averaging, histogram matching, and solvent flattening using DM (CCP4, 1994), allowing building of the α-helical segments of IκBα and placement of one of the two RHRn p65 domains using the program O (Jones et al., 1991). Native data set B was then used for further model building and refinement. The position of the second RHRn p65 domain was determined to be in an orientation in the complex similar to the first RHRn, but with fewer contacts to other domains, and is thus not well ordered. The model was refined using conjugate gradient minimization and both Cartesian and torsion-angle simulated annealing with the programs X-PLOR and CNS (Brünger, 1992; Rice and Brünger, 1994; Pannu and Read, 1996).

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Brookhaven Protein Data Bank ID Code

The ID code for the structure described in this manuscript is 1nfi, and the coordinates are available at http://crystal.harvard.edu/harrison/coordinates/.