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The Crystal Structure of the IκBα/NF-κB Complex Reveals Mechanisms of NF-κB Inactivation

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Summary

IκBα regulates the transcription factor NF-κB through the formation of stable IκBα/NF-κB complexes. Prior to induction, IκBα retains NF-κB in the cytoplasm until the NF-κB activation signal is received. After activation, NF-κB is removed from gene promoters through association with nuclear IκBα, restoring the preinduction state. The 2.3 Å crystal structure of IκBα in complex with the NF-κB p50/p65 heterodimer reveals mechanisms of these inhibitory activities. The presence of IκBα allows large en bloc movement of the NF-κB p65 subunit amino-terminal domain. This conformational change induces allosteric inhibition of NF-κB DNA binding. Amino acid residues immediately preceding the nuclear localization signals of both NF-κB p50 and p65 subunits are tethered to the IκBα amino-terminal ankyrin repeats, impeding NF-κB from nuclear import machinery recognition.

Introduction

The survival of organisms depends on their ability to react to stimuli with the appropriate complement of genetically encoded responses. Transcription factors, such as NF-κB, activate the expression of specific genes in order to evoke the required cellular response. NF-κB itself is tightly regulated through its association with IκBα. Acting in concert, these two proteins turn on and off vital genes required for the immune response and inflammation, cellular growth and differentiation, cell adhesion, and apoptosis (for reviews, Baeuerle and Henkel, 1994; Siebenlist et al., 1994; Baeuerle and Baltimore, 1996; Baldwin, 1996).

The mammalian Rel/NF-κB family of inducible transcription factors is comprised of five structurally related polypeptides, p50, p65 (RelA), p52, c-Rel, and RelB, which associate to form transcriptionally competent homo- and heterodimers. Of these dimers, the p50/p65 heterodimer (prototypical NF-κB) is the most abundant and biologically active.

Polypeptides of the Rel/NF-κB family are defined by an approximately 300 amino acid long Rel homology region (RHR). All the residues necessary for subunit dimerization, sequence-specific DNA binding, nuclear localization, and inhibitor binding are contained within the RHR. Regions beyond the RHR are primarily responsible for the transactivation potential of these transcription factors. Crystal structures containing the RHR of three Rel/NF-κB family polypeptides complexed with various DNA targets have been determined (Ghosh et al., 1995; Müller et al., 1995; Cramer et al., 1997; Chen et al., 1998a, 1998b). These structures indicate that the RHR exhibits a tripartite organization. The amino-terminal 180 amino acids fold into an immunoglobulin-like domain. A short (10 amino acids) flexible linker connects this amino-terminal domain to a second immunoglobulin-like domain of approximately 100 amino acids in length. All dimerization contacts are mediated through this second domain, referred to as the dimerization domain. The carboxy-terminal 13 amino acids (NLS polypeptide), the final four of which contain a basic nuclear localization sequence (NLS), are disordered in the crystal structures, suggesting flexibility. DNA base-specific contacts are made by loops emanating from the amino-terminal domain, while nonspecific DNA ribo-phosphate backbone interactions involve loop residues from both the amino-terminal and dimerization domains.

In its resting state, the p50/p65 heterodimer exists in a stable cytosolic complex with a member of the inhibitor κB (IκB) family (Figure 1a) of transcription factor inhibitor proteins (for reviews, Baeuerle and Baltimore, 1988; Verma et al., 1995; Baeuerle and Baltimore, 1996; Baldwin, 1996). Inducers of NF-κB, including bacterial and viral products, inflammatory cytokines, reactive oxygen, and ultraviolet light, activate a kinase complex specific for IκB (Stancovski and Baltimore, 1997). Activated IκB kinase (IKK) triggers the sequence-specific phosphorylation and ubiquitination of the complex-associated IκB molecule, resulting in the rapid degradation of IκB through a ubiquitin-proteosome conjugated pathway (Ghosh and Baltimore, 1990; Traenckner et al., 1994; Chen et al., 1995). Removal of IκB activates the NF-κB NLSs so that NF-κB rapidly translocates into the nucleus, binds to select gene promoters in a sequence-specific manner, and activates gene transcription.

The six IκB family proteins in higher eukaryotes, IκBα, IκBβ, p105, p100, IκBϵ (Whiteside et al., 1997), and Bcl-3, contain a centrally located ankyrin repeat domain (ARD). Ankyrin repeats are 33 amino acid modules originally identified in the human erythrocyte protein ankyrin. Ankyrin repeats have since been identified in numerous proteins with diverse functions (Michaely and Vennett, 1992; Bork, 1993). Amino-terminal to the ARD, the signal receiving domain (SRD) contains amino acid residues that accept the phosphorylation and ubiquitination activation signals. Though a vital component of NF-κB activation, the SRD does not appear to physically participate in IκB/NF-κB complex formation (Hatada et al., 1992; Jaffray et al., 1995; Sun et al., 1996). The segment carboxy-terminal to the ARD is rich in the amino acids proline, glutamic acid, serine, and threonine (PEST). The PEST sequence has long been recognized as an element common to proteins exhibiting rapid turnover in the cell (Rogers et al., 1986). Considerable biochemical evidence suggests, however, that the carboxy-terminal PEST region of IκBα also participates with the ARD in forming the stable IκBα/NF-κB complex (Ernst et al.,

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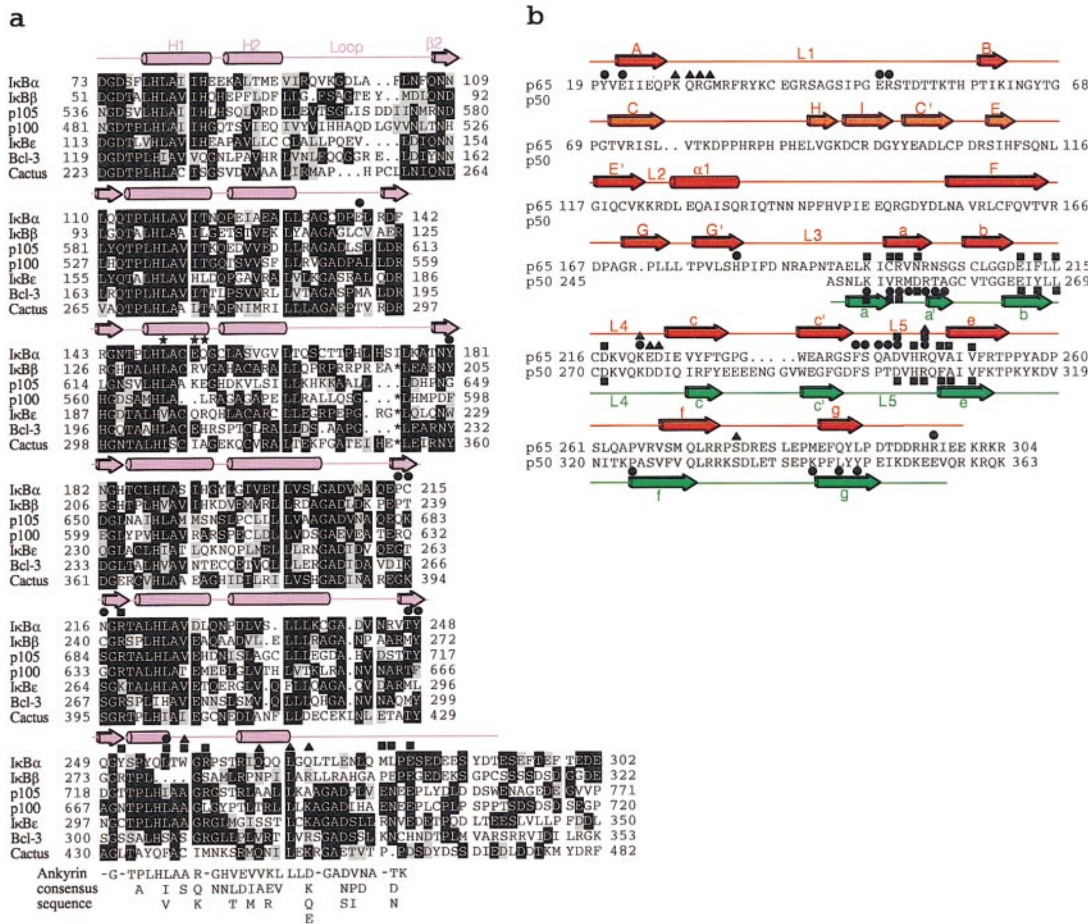

Figure 1. Primary Sequence and Secondary Structure of IκBα and NF-κB p50 and p65 Subunits

(a) Sequence alignment of the human IκB family proteins and *Drosophila* homolog Cactus. Black boxes indicate identities, and hatched boxes highlight conservative changes. Secondary structure elements, as determined in the complex structure, are indicated above the sequence of IκBα. Arrows indicate β strands, and cylinders represent α helices. H1 and H2, Loop, and β2 represent the conserved structural elements of the ankryin repeat. Asterisks in the loop of ankyrin repeat 3 signify insert regions (see text). Specific residues involved in interactions with NF-κB are indicated by the following symbols over the amino acid single letter code: circles, contact amino acid residues in p50 dimerization domain; squares, p65 dimerization domain; triangles, p65 amino-terminal domain; stars, p65 NLS polypeptide.

(b) Sequences of the murine p65 RHR and murine p50 dimerization domain and NLS polypeptide used in IκBα/NF-κB complex structure. Names of secondary elements and five DNA-contacting loops follow conventions established previously (Ghosh et al., 1995). Amino acid contacts are indicated by the following symbols: circles, contact IκBα; squares, participate in NF-κB dimer formation; triangles, NF-κB intramolecular interactions.

1995; Malek et al., 1998). Casein kinase II constitutively phosphorylates serine and threonine residues in this region (McElhinny et al., 1996; Schwarz et al., 1996).

IκBα was discovered as an activity that dissociates preformed NF-κB/DNA complexes in vitro (Baeuerle and Baltimore, 1988; Zabel and Baeuerle, 1990). It was shown later that IκBα exerts its primary inhibitory function by physically masking the NF-κB NLSs (Zabel and Baeuerle, 1990; Beg et al., 1992; Henkel et al., 1992). Analysis of its gene promoter and mRNA synthesis revealed that transcription of IκBα itself is regulated by NF-κB (Brown et al., 1993; de Martin et al., 1993; Sun et al., 1993). Subsequent studies indicated that in the postinduction stage and in the absence of cytosolic NF-κB, newly translated IκBα localizes to the nucleus where it can remove NF-κB from gene promoters (Zabel et al., 1993; Arenzana-Seisdedos et al., 1995). A purported IκBα nuclear export sequence (NES) is then thought to signal for active shuttling of the IκBα/NF-κB complex to the cell cytosol, restoring the preinduction state (Arenzana-Seisdedos et al., 1997). This feedback inhibition mechanism represents the second mode in which IκBα controls NF-κB activity. Thus, the IκBα molecule regulates both the activation and inactivation of gene transcription through its association with NF-κB.

To clarify the structural basis for inhibition of both nuclear entry and DNA binding of NF-κB by IκBα, we have determined the crystal structure of a murine NF-κB p50/p65 heterodimer in complex with human IκBα. The crystal structure illustrates how IκBα/NF-κB interactions promote cytoplasmic retention and transcriptional inactivation of NF-κB. Amino acid residues vicinal to

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

Crystal[a]	Native 1	Native 2	PMA	$K_2[PtCl_4]$
X-ray beamline[b]	Home source	X25	Home source	X4A
Maximum resolution (Å)	3.1	2.3	3.1	3.0
Total observations	125,422	160,828	74,868	124,258
Unique reflections	12,808	28,014	12,575	12,760
Completeness (%)	98.9 (98.1)	91.2 (59.4)	97.2 (97.0)	83.2 (52.1)
R_{sym}[c] (%)	10.8 (43.2)	4.3 (22.1)	8.3 (30.1)	8.3 (47.3)
I/σ	6.8 (2.4)	15.1 (2.5)	8.8 (2.5)	13.2 (2.0)
No. of metal-binding sites			6	7
R_{iso}[d] (%)			17.6	27.3
Phasing power[e] (centric/accentric)			0.88/0.65	0.80/0.57

Refinement (with Native 2 Data Set at 6.0–2.3 Å)

	3 σ Data	All Data	Rmsd from Ideal Values	
			Bonds (Å)	Angles (°)
R_{cryst}[f] (%)	22.1	23.8		
R_{free}[g] (%)	27.7	28.7	0.007	1.39

Data for the outermost resolution shell are given in parentheses.
[a] PMA-phenylmercuric acetate (1 mM for 6 hr), $K_2[PtCl_4]$ (0.5 mM for 3 hr).
[b] X25 and X4A: National Synchrotron Light Source beamlines X25 and X4A, Brookhaven National Laboratory.
[c] $R_{sym} = \Sigma|I_h - \langle I_h\rangle|/\Sigma I_h$, where $\langle I_h\rangle$ is the average intensity over symmetrically equivalent measurements.
[d] $R_{iso} = \Sigma|F_{PH} - F_P|/\Sigma F_P$, where F_{PH} and F_P are the derivative and native structure factor amplitudes.
[e] Phasing power $= \Sigma|F_H|/\Sigma||F_{PH}obs| - |F_{PH}\ calc||$.
[f] $R_{cryst} = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$, where summation is over data used in the refinement.
[g] R_{free} is the same calculation including only the 5% of data excluded from all refinements.

both the p50 and p65 NLSs are tethered to the first two ankyrin repeats of IκBα, suggesting a mechanism for cytoplasmic retention of NF-κB. In the presence of IκBα, the p65 subunit of NF-κB undergoes a profound conformational change. Adoption of this closed conformation by NF-κB impedes DNA binding through the occlusion of basic DNA-contacting surfaces.

Results and Discussion

Structure Determination and Overall Architecture of the Complex

Complex cocrystals containing the p50 and p65 subunits of NF-κB and IκBα were obtained after extensive screening with truncated fragments of all three components. Biochemical characterization of binding affinities for deletion constructs of p50, p65 (Figure 1b), and IκBα identified the minimal parts necessary for stable complex formation and native activity. These studies clearly show that the amino-terminal SRD of IκBα and the p50 amino-terminal domain do not contribute to IκBα/NF-κB complex binding affinity (Malek et al., 1998). The protein fragments used in complex cocrystallization include amino acid residues 245–363 of murine p50 (p50ddNLS), residues 19–304 of murine p65 (p65RHR), and residues 67–302 of human IκBα. The complex crystallized in a centered monoclinic space group with one complex (MW 72 kDa) per asymmetric unit. Initial crystallographic phases of the IκBα/NF-κB complex were determined at 3.1 Å resolution by combining molecular replacement, multiple isomorphous replacement, and solvent modification methods. The structure has been refined with 2.3 Å data to a crystallographic R factor of 22.1% and free R factor of 27.7% with excellent stereochemistry. Table 1 summarizes the results of data collection and phase determination and reports current refinement statistics.

A structural representation of the complex is presented in Figures 2a–2c. The most distinguishing feature of the complex is the intimate nature of the association between the NF-κB heterodimer and IκBα. The cylindrical-shaped ARD of IκBα stacks above the NF-κB dimer interface so that its axis is antiparallel with respect to the amino and carboxyl termini of the p50 and p65 dimerization domains (Figures 2a–2c). This compact assembly rests atop the amino-terminal domain of p65 in an orthogonal orientation. While the three domains, the p50 dimerization domain, the p65 dimerization domain, and the IκBα ARD, project roughly from the plane of the p65 amino-terminal domain base, the ARD of IκBα extends above the cleft formed at the p50/p65 dimer interface on the other end. The first and second ankyrin repeats constitute this IκBα protrusion. The p50 and p65 carboxy-terminal segments containing the NLSs extend from the top of the dimerization domains and contact opposite faces of the first two ankyrin repeats. The carboxy-terminal PEST region of IκBα at the base of the complex fills the cavity created by the interface of the p65 amino-terminal domain and p50 dimerization domain.

IκBα Structure

The six repeating units of the IκBα ARD resemble those of other ankyrin repeat–containing proteins such as 53BP2 (Gorina and Pavletich, 1996), the cyclin-dependent protein kinase inhibitors $p18^{INK4c}$ (Venkataramani et al., 1998) and $p19^{INK4d}$ (Luh et al., 1997), and the β subunit of transcription factor GABP (Batchelor et al., 1998). Each repeat contains two closely packed α helices followed by a loop and a tight hairpin turn (Figure 2d). Helix pairs stack between those of the preceding and following units with roughly 11 Å spacing. After the second helix in each repeat, a loop of variable length extends perpendicular to the helices. Each loop closes

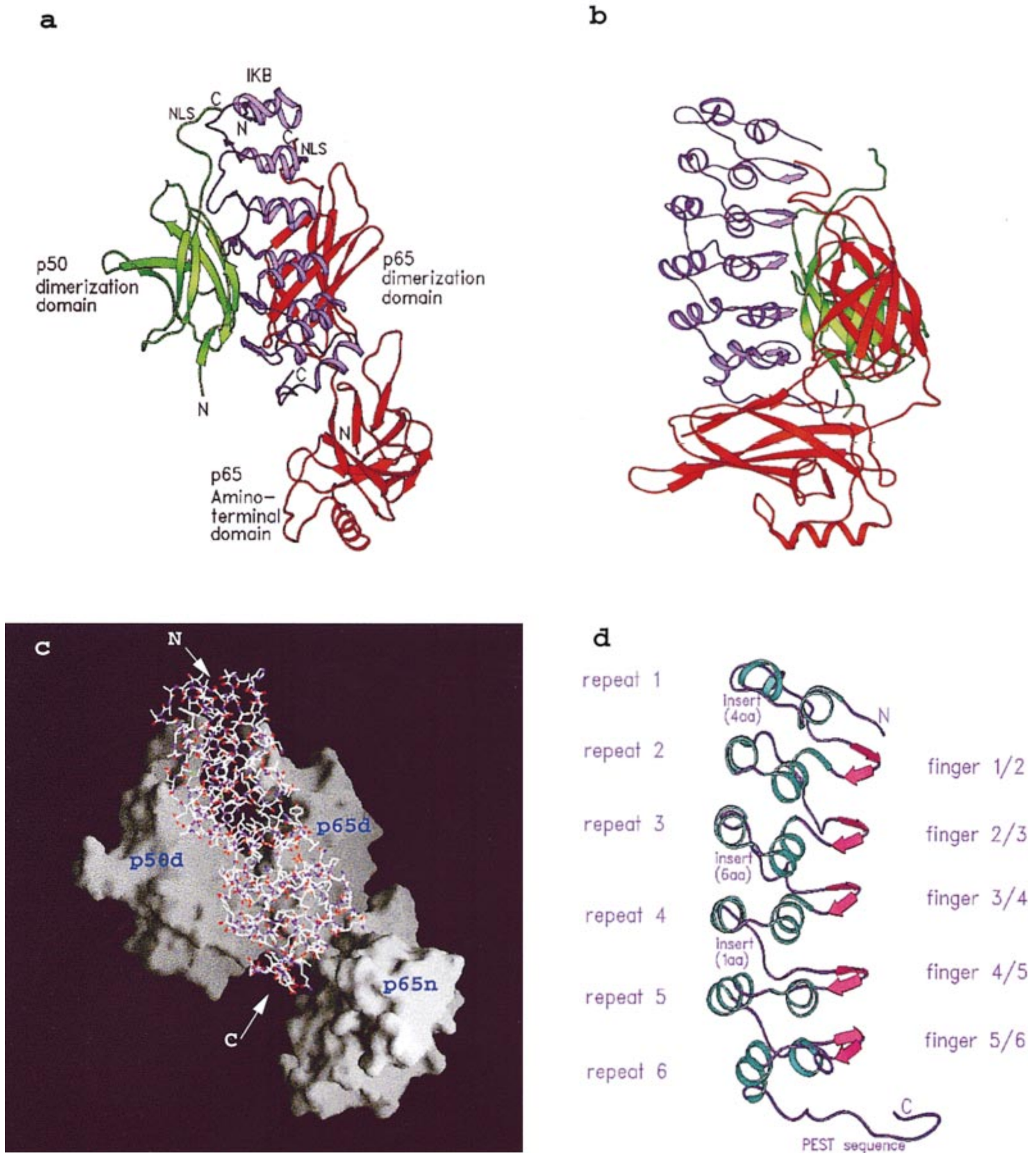

Figure 2. Three-Dimensional Structure of the IκBα/NF-κB Complex

(a) Ribbon diagram of the complex aligned to the dimer axis of NF-κB. IκBα is represented in purple; the p50 and p65 subunits of NF-κB are green and red, respectively. This and all other ribbon diagrams were generated using SETOR (Evans, 1993).

(b) Complex rotated 90° about the NF-κB dimer axis.

(c) The IκBα/NF-κB complex is shown with NF-κB depicted as a molecular surface. IκBα sits across the dimer platform, with its ARD extending above the dimer interface and its carboxy-terminal PEST region filling the cleft created by the p65 amino-terminal and p50 dimerization domains. The amino and carboxyl termini of IκBα are indicated C and N, respectively. The p50 and p65 dimerization domains are labeled p50d and p65d, respectively, and the amino-terminal domain of p65 is labeled p65n. This and all other figures with molecular surfaces were generated using GRASP (Nicholls, 1992).

(d) Three-dimensional structure of IκBα shown in a ribbon diagram. The molecule contains six ankyrin repeats followed by a carboxy-terminal PEST region. With the exception of ankyrin repeats 1 and 6, each repeat begins with a short β strand (in magenta) followed immediately by two α helices (in teal), a loop region of variable length, and a second short β strand (in magenta). Variations in the length of loop regions in ankyrin repeats 1, 3, and 4 are labeled insert. Each "finger" results from a β hairpin created by the carboxy-terminal β strand of one repeat and the amino-terminal β strand of the successive repeat.

with a β hairpin formed by short β strands from the carboxy-terminal amino acid residues of the repeat and the amino-terminal residues of the successive repeat. The overall arrangement resembles five finger-like projections extending from the concave side of a bent cylinder. The first and last ankyrin repeats contribute only one β strand each, and as a result, n ankyrin repeats create n-1 finger-like projections. Throughout this paper the notation "finger 1/2" will be used to indicate the β hairpin that connects ankyrin repeats 1 and 2 of IκBα (Figure 2d).

Sequence comparison against the ankyrin consensus

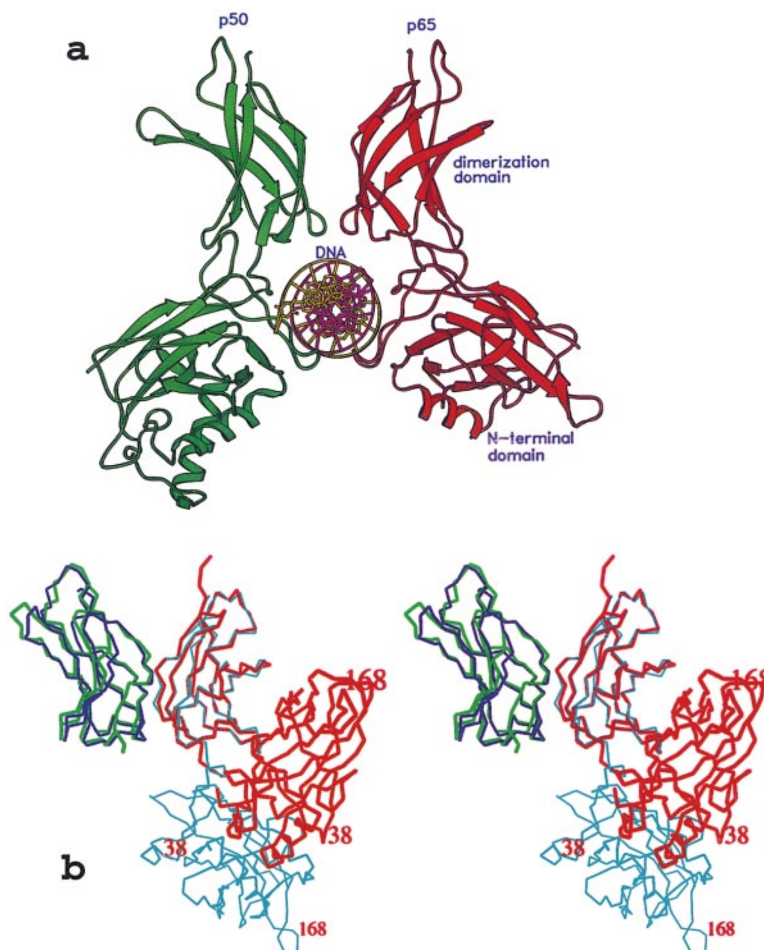

Figure 3. NF-κB p50/p65 Heterodimer in Its Open and Closed Conformations

(a) NF-κB p50/p65 heterodimer bound to κB DNA target (Chen et al., 1998a) exhibits its open conformation. Note that the amino-terminal domain of p50 subunit is present in this DNA-bound complex.

(b) Stereoview of the p50/p65 heterodimer in its DNA-bound open conformation (p50dd in blue, p50 amino-terminal domain not present, and p65RHR in cyan) and IκBα-bound closed conformation (p50dd in green and p65RHR in red) overlayed by superposition of the dimerization domains. The p65 amino-terminal domain rotates 171° and translates 38 Å between the two conformations.

shows that repeats 1, 3, and 4 of IκBα are longer than 33 amino acids (Figure 1a). The inserts fall in the loop regions, which also represent the areas of least homology between IκBα and other ankyrin repeat–containing proteins. Variability between the ankyrin loops is more pronounced in other IκB family members. For example, the third repeats of IκBβ and the *Drosophila* homolog Cactus contain loop region inserts of 47 and 30 amino acids, respectively. Insertions in the ARD loop regions do not disrupt the integrity of the core of the ankyrin repeat structure, since these insertions reside on the convex back side of the domain away from the NF-κB binding surface.

Ankyrin repeat 6 of IκBα loses its homology to the consensus sequence after its second helix. Of the 22 amino acids carboxy-terminal to the sixth repeat, the last 11 are disordered. Amino acids 280–291 (the acidic PEST sequence) assume a serpentine conformation devoid of secondary structure.

NF-κB Structure

Conformational Change of the p65 Subunit

The most intriguing feature of the complex structure is the remarkable change in conformation adopted by the p65 subunit of NF-κB in the presence of IκBα. Compared to its DNA-bound "open" conformation (Figure 3a), the IκBα-bound p65 amino-terminal domain rotates almost 180° about its long axis and translates 38 Å toward its carboxy-terminal dimerization domain (Figure 3b). Root-mean-square (rms) deviation for superposition of the DNA-bound and IκBα-bound p65 amino-terminal domains is only 1.0 Å, indicating en bloc movement of the entire domain. Therefore, it appears that this movement is afforded entirely by the flexible ten amino acids linker connecting the p65 amino-terminal and dimerization domains. The relatively high temperature factor of the NF-κB p65 subunit amino-terminal domain (51 $Å^2$) and the flexibility of the IκBα carboxy-terminal PEST sequence suggest that the IκBα-bound p65 subunit conformation may differ in solution.

Small movements of the p65 amino-terminal domain within the context of the open conformation have been observed before. These motions allow for variability in DNA sequence recognition (Chen et al., 1998b). Similarly, conformational change by the p65 subunit of NF-κB in the presence of IκBα permits optimal IκBα/NF-κB complex formation. Adoption of this closed conformation allows for allosteric inhibition of NF-κB DNA binding. Moreover, the conformational change is likely to impede phosphorylation of Ser-276 by cAMP-dependent protein kinase (PKA), which is required for full transcriptional activity of NF-κB (Zhong et al., 1998).

NF-κB Dimer Interface

The overall architecture of the p50 and p65 subunit dimer interface is similar to that of the heterodimer–DNA complex. Fourteen residues from each subunit participate in the dimer interface, which is dominated by van der Waals interactions. Three homologous amino acid pairs located at the core of the interface, *Tyr-267*/Phe-213, *Leu-269*/Leu-215, and *Val-310*/Val-251 (p50 residues shown in italics), mediate several critical contacts with residues of the opposing subunit. Other pairs of residues that participate in dimer interface formation are *Lys-249*/Lys-195, *Val-251*/Cys-197, *Arg-252*/Arg-198, *Asp-254*/Asn-200, *Glu-265*/Glu-211, *Asp-271*/Asp-217, *Asp-302*/Asp-243, *His-304*/His-245, *Arg-305*/Arg-246, *Phe-307*/Val-248, and *Ala-308*/Ala-249. Despite the similarities, the detailed chemical nature of the IκBα-bound p50/p65 dimer interface reveals alterations from its DNA-bound conformation. One notable difference in the IκBα-bound complex is the involvement of the guanidinium groups of *Arg-305* and Arg-246 in salt bridge formation with Asp-217 and *Asp-271*, respectively. In the DNA-bound conformation, these arginine side chains participate in nonspecific DNA ribo-phosphate backbone contacts. We also observe an overall slight opening of the dimer interface upon IκBα binding. The most striking change, however, is the loss of the *Asp-254*/Asn-200 hydrogen bond, one of the most critical interactions in discriminating subunit dimerization specificity among NF-κB dimers (Huang et al., 1997; Chen et al., 1998a).

IκBα/NF-κB Heterodimer Interactions

Interactions between the NF-κB p50/p65 heterodimer and IκBα are extensive and bury 3800 Å^2 of solvent-accessible surface area. Interestingly, the NF-κB-DNA complex excludes a similar surface area (3754 Å^2). All five structurally independent parts of NF-κB—three from p65RHR and two from p50ddNLS—contact IκBα. The interactions of IκBα with NF-κB fall into three categories: recognition of the NF-κB dimer interface, p65 amino-terminal domain binding, and anchoring the NLS polypeptides.

Recognition of the NF-κB Dimer Interface

The dimerization domain of NF-κB is the primary site of recognition by IκBα. 2361 Å^2 of the total 3800 Å^2 solvent-excluded surface area is derived from the NF-κB dimerization domain–IκBα ARD interface. IκBα orients itself such that fingers 3/4, 4/5, and 5/6 contact the p50 subunit (Figure 4a). Amino acid residues Tyr-181 and Asn-182 extend from finger 3/4 to participate in multiple contacts with side chains arising from strands a, a′, and g of p50. Tyr-181, in particular, forms hydrogen bonds with *Lys-249* and *Arg-255*, stacks with *Tyr-348* and makes multiple van der Waals contacts with *Ala-257*, *Pro-324*, and *Leu-346*. Subcellular localization and in vitro affinity measurements of Tyr-181 to Ala site-directed mutants confirm the role of this residue in IκBα/NF-κB complex formation (L. Sengchanthalangsy and G. Ghosh, unpublished result; N. Rice, personal communication). Likewise, amino acid residues Pro-214, Cys-215, Asn-216, Thr-247, and Tyr-248 from fingers 4/5 and 5/6 also interact with the p50 subunit. The participation of IκBα ankyrin fingers in recognizing p50 is analogous to the interactions between the ankyrin repeat–containing β subunit of transcription factor GABP with its α subunit (Batchelor et al., 1998). Amino acids *Val-251* and *Arg-252* of p50 participate in both the dimer interface and interactions with IκBα.

The alignment of IκBα on the NF-κB dimerization platform is tilted slightly toward the p50 subunit allowing for fewer interactions between the ARD and p65 dimerization domain. Loop five (L5 in Figure 1b) of the p65 dimerization domain and the last two ankyrin repeats of IκBα contribute significantly to the IκBα-p65 dimerization domain interactions. Most of these contacts are electrostatic in nature including salt bridges between Asp-243 of p65 and Arg-218 of IκBα (Figure 4b) and between Arg-246 of p65 and Glu-282 of IκBα. The NF-κB dimer platform lacks a binding pocket specific for the ARD of IκBα. Rather, the two factors interact by burying extensive, relatively planar surfaces.

p65 Amino-Terminal Domain Binding

IκBα uses its sixth ankyrin repeat and acidic carboxy-terminal PEST sequence to bind the amino-terminal domain of p65. Amino acid residues Tyr-20, Glu-22, Glu-49, Arg-50, Arg-158, and His-181 from three different regions of the p65 amino-terminal domain contact IκBα residues Trp-258, Gln-278, Glu-275, Pro-281, Met-279, and the backbone nitrogen of Gly-259 (Figure 5a). Besides these specific contacts, a host of long-range electrostatic interactions are also made between the acidic residues of IκBα and basic p65 amino-terminal domain residues (discussed below).

Anchoring the NLS Polypeptides

Our structure reveals that the p65 NLS polypeptide bridges the 15 Å distance between the top of the p65 dimerization domain and ankyrin repeats 1 and 2 of IκBα. The interaction (Figure 5b) buries 466 Å^2 solvent-accessible surface area. Arg-297 of p65 anchors the NLS polypeptide to the loop region of ankyrin repeats 2 and 3 on the convex face of IκBα. p65 residues 301–304, containing the basic NLS, exhibit scattered electron density in the vicinity of this convex face of ankyrin repeats 1 and 2 of IκBα. The NLS polypeptide of p50, on the other hand, remains close to the first two fingers and the amino terminus of IκBα. Only 175 Å^2 of surface area is excluded as a result of this alignment. Similar to the p65 NLS, the final six amino acid residues of p50 also display broken electron density. These observations are consistent with in vitro biochemical experiments which show that p50 and p65 NLS polypeptides contribute only marginally to the overall binding affinities of IκBα/NF-κB complexes (Malek et al., 1998).

Inhibition of DNA Binding

Inhibition of NF-κB DNA binding by IκBα arrests NF-κB-mediated activation of transcription. IκBα inhibits DNA binding through a complex series of interactions. NF-κB binding of IκBα juxtaposes the sixth ankyrin repeat of IκBα and the bottom of the p65 dimerization domain, resulting in the creation of a continuous, planar negatively charged surface. The placement of added negative charge on the p65 subunit recruits the highly basic DNA-contacting face of the p65 amino-terminal domain to within close proximity of this negatively charged surface. This stable electrostatic interface measures 43 Å

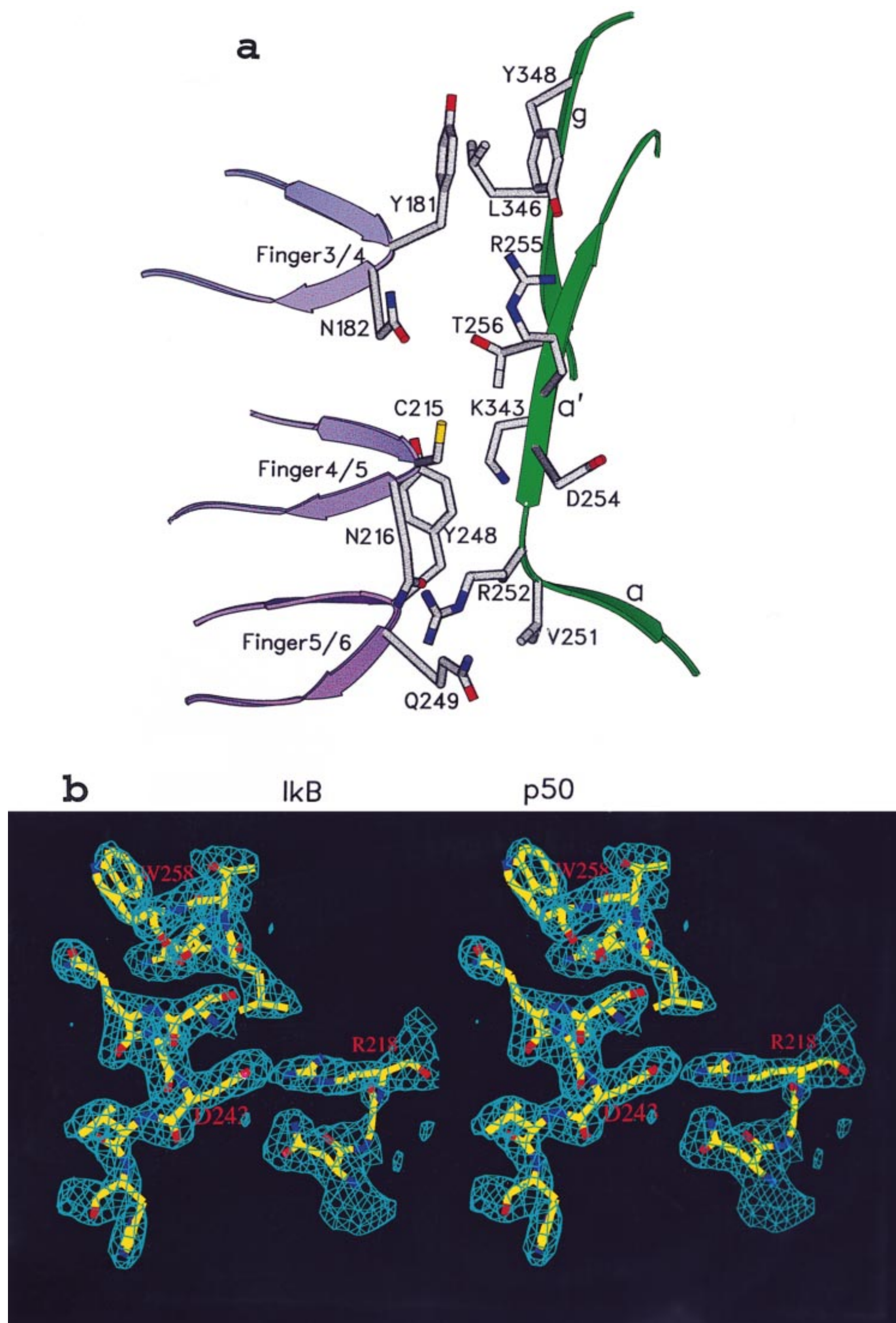

Figure 4. IκBα Interactions with the NF-κB Dimer Platform

(a) Fingers 3/4, 4/5, and 5/6 (in purple) form hydrogen bonds and participate in van der Waals interactions with side chains from β strands a, a′, and g of the p50 dimerization domain (in green). Amino acid single letter codes are used.

(b) Stereoview of a representative portion of the electron density map at a contour level of 1.8 σ depicting the interactions of IκBα with the p65 dimerization domain. Arg-218 from the loop of IκBα ankyrin repeat 5 participates in electrostatic interactions with Asp-243 from loop 5 of the p65 dimerization domain. Also shown is the main-chain hydrogen bond between Trp-258 of IκBα and Gln-241 of p65.

long and 32 Å wide and buries 1535 $Å^2$ solvent-accessible surface area (Figure 5c). Amino acid residues Glu-222, Asp-223, Glu-225, and Asp-243 from the p65 dimerization domain and Glu-275, Glu-282, and Glu-284 of IκBα combine to form the acidic surface. The p65 amino-terminal domain contributes Lys-28, Arg-30, Arg-33, Arg-35, Arg-50, Lys-62, Lys-79, Arg-158, His-181, and Arg-187 to the basic face. Arg-33 and Arg-35, two affinity- and specificity-determining DNA-binding residues from loop L1 of the p65 subunit, participate through long-range electrostatic interactions. Further stabilization of this interface arises from other polar and van der

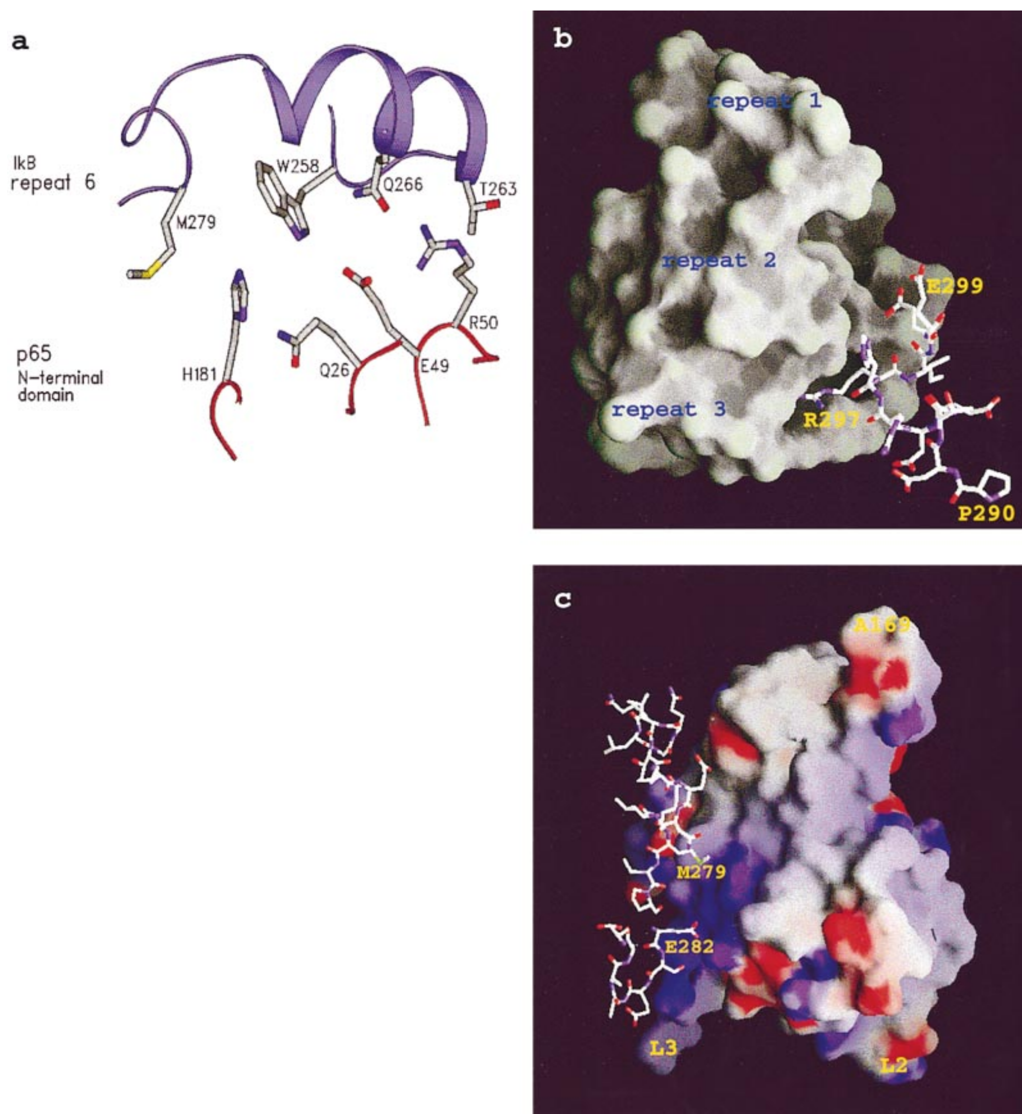

Figure 5. IκBα Interactions with the p65 Amino-Terminal Domain and NLS Polypeptides

(a) Residues from the sixth ankyrin repeat of IκBα (in purple) contact side chains from the p65 amino-terminal domain (in red). Amino acid single letter codes are used.

(b) The interaction of the p65 NLS polypeptide (skeleton) is shown with IκBα ankyrin repeats 1, 2, and 3 depicted as a molecular surface. The orientation is the same as in Figure 2a.

(c) Molecular surface representation of the p65 amino-terminal domain according to local electrostatic surface potentials (blue, positive; red, negative). Skeleton representation of the amino acids 268–288 (part of sixth ankyrin repeat and PEST sequence) of IκBα contact the basic patch (blue) of the p65 amino-terminal domain. Orientation is as in Figure 2b. DNA-binding loops 2 and 3 are labeled L2 and L3, respectively. This is only part of the extensive network of interacting electrostatic surfaces formed between the amino-terminal and dimerization domains of p65 and IκBα.

Waals contacts of IκBα residues Met-279, Pro-281, Trp-258, and Gln-278 and the amino-terminal domain of p65, as previously described.

Besides interacting directly with the DNA-binding residues of NF-κB, IκBα permits allosteric regulation of the p65 subunit by locking it into a closed conformation. Furthermore, the acidic carboxy-terminal PEST sequence of IκBα is involved in neutralizing basic DNA-contacting amino acids from the bottom of the p50 dimerization domain. This model of NF-κB DNA-inhibitory binding by IκBα implies that the addition of negative charge to IκBα might lead to more effective inhibition of NF-κB DNA binding by IκBα. Indeed, biochemical results indicate that the phosphorylation of the PEST region at Ser-283, Ser-288, Ser-293, and Thr-291 by casein kinase II renders IκBα a more effective inhibitor of NF-κB DNA binding (McElhinny et al., 1996).

It has been shown that unphosphorylated IκBβ does not inhibit NF-κB DNA binding but instead forms a stable complex with DNA-bound NF-κB (Thompson et al., 1995; Tran et al., 1997). Significant differences in the amino acid sequences of the sixth repeats in IκBα and IκBβ

could explain this difference. Several key amino acids that contact the amino-terminal domain of p65, Trp-258, Met-279, Pro-281, and Gln-278, are unique to IκBα. These residues could play an essential role in stabilizing the closed conformation of p65. Phosphorylation of some or all of the serines and threonines within the sixth repeat and PEST sequence of IκBβ may serve to compensate for the lack of these interactions and force the observed p65 subunit conformational change.

Based on our structure, ankyrin repeats 1 and 2 of IκBα appear to participate in the fewest interactions with NF-κB. It comes as little surprise, therefore, that a virus-encoded protein that shows significant sequence homology to ankyrin repeats 3 through 6 of IκBα attenuates the inflammation response of macrophages by inhibiting NF-κB activity (Powell et al., 1996; Revilla et al., 1998). We propose that these regions of IκBα alone are sufficient to promote adoption of the closed conformation and, thus, interfere with NF-κB DNA binding.

Masking of the NF-κB NLS

Although it has long been speculated that IκBα sequesters NF-κB in the cytosol by masking the NLSs (KRKR in p65 and KRQK in p50), the precise mechanism of retention has not been revealed (Beg et al., 1992; Henkel et al., 1992). Despite widely held belief, evidence supporting the direct interaction between the NLSs of NF-κB and IκBα has never been obtained. For example, Zabel and others (1993) clearly showed that IκBα binding blocks the access of antibodies specific for peptides overlapping the NLSs of p50 and p65. Our own biochemical characterization of IκBα/NF-κB complexes (Malek et al., 1998) revealed small contributions to binding affinities due to direct contacts between IκBα and the carboxy-terminal 13 amino acids of p50 RHR and p65 RHR. Both of these studies fail, however, to distinguish the basic NLS (the four amino acid residues that convey nuclear translocation to NF-κB) from the flexible NLS polypeptide (the 13 amino acid segments that do not assume an ordered structure in the DNA-bound conformation and which contain the basic NLS). Indeed, as the IκBα/NF-κB complex crystal structure reveals, IκBα interactions with the NF-κB NLS polypeptides occur through amino acid residues vicinal to the actual NLSs. This observation serves to explain both the altered affinity for NF-κB mutants lacking the NLS polypeptide regions and the inability of antibodies to recognize these sequence when bound to IκBα.

Numerous other studies have identified varied and conflicting regions of NF-κB and IκBα as necessary elements for NF-κB cytosolic retention. One laboratory has reported the involvement of the amino-terminal SRD of IκBα in masking the NLS of p50 and c-Rel homodimers by observing subcellular localization of Rel/NF-κB and IκBα in transfected cells (Latimer et al., 1998). Others report that this same region is not involved in complex formation (Inoue et al., 1992; Jaffray et al., 1995). Our own results indicate that the presence or absence of the IκBα SRD does not alter binding affinities for p50 homodimers or the p50/p65 NF-κB heterodimer (Malek et al., 1998). There are many factors unique to the IκBα/NF-κB transcription factor system that contribute to this confusion. First of all, the biological activities of the Rel/NF-κB homo- and heterodimers are linked to their subcellular concentrations and localization. For example, at physiologic levels the p50 homodimer escapes interaction with IκBα and localizes to the nucleus where it acts as a transcriptional repressor (Franzoso et al., 1992). The discovery of nuclear IκBα (Zabel and Baeuerle, 1990; Arenzana-Seisdedos et al., 1995) and the possibility of active nuclear exit by both NF-κB and IκBα (Arenzana-Seisdedos et al., 1997; S. Taylor, unpublished data) further complicate the interpretation of biochemical and cell biological data. Moreover, because overexpression of Rel/NF-κB polypeptides leads to homodimer formation, none of these studies adequately addresses the interaction of IκBα with the p50 and p65 NLSs in the context of the p50/p65 NF-κB heterodimer.

Based on this structure, IκBα recognizes the sequences flanking the NF-κB NLSs and appears to inhibit nuclear localization by sterically impeding NLS access to the nuclear import machinery. This mode of interaction is in contrast to that of the SV40 large T antigen NLS with importin. The cocrystal structure of the nuclear import protein karyopherin α in complex with two SV40 NLS polypeptides shows that the protein directly binds NLSs in an extended conformation to accommodate both van der Waals and electrostatic interactions (Conti et al., 1998). It is, however, also possible that direct interactions of IκBα with the NLS could require regions of the polypeptides carboxy-terminal to the NLSs. The independent solution of a similar IκBα/NF-κB complex crystal structure by Jacobs and Harrison indicates that this is indeed the case (1998 [this issue of *Cell*]).

Finally, it is important to note that Govind and coworkers (1996) have shown in the *Drosophila* system that nuclear translocation of the NF-κB homolog Dorsal is blocked by deletion of the 39 amino-terminal residues from the Dorsal RHR. This suggests that along with the NLS, this segment also plays a direct role in nuclear translocation. The corresponding residues in p65RHR (amino acids 19–57) contact IκBα as well as its own carboxy-terminal domain. Together, anchoring of the NF-κB NLSs and occlusion of the p65 amino-terminal DNA-binding surfaces through association with IκBα may explain the IκBα/NF-κB complex cytoplasmic retention mechanism.

Specificity of Interaction

Fundamental to NF-κB/IκBα regulation of transcription is the selectivity of IκBα toward p65- and c-Rel-containing homo- and heterodimers including the NF-κB p50/p65 heterodimer. As a consequence of this selectivity, the p50 homodimer, which bears no inherent transactivation potential, escapes IκBα regulation and localizes in the nucleus, where it acts to repress transcription in unstimulated cells (Franzoso et al., 1992). Biochemical characterization of IκBα binding specificity has not been studied extensively. Recently, we have shown that IκBα does in fact bind p50 homodimers but with a 60-fold lower affinity than for the p50/p65 heterodimer. Homodimers of p65, however, are bound by IκBα with an affinity 27-fold lower than that of the heterodimer (Malek et al., 1998). These differences in binding affinities could

originate from all three interacting segments of NF-κB: the amino-terminal domain, the dimerization domain, and the NLS polypeptides. Consistent with biochemical characterization of the complex (Govind et al., 1996; Latimer et al., 1998), this structure reveals limited interaction between IκBα and the NF-κB NLS polypeptides. Therefore, these sequences are unlikely to contribute substantially to observed differences in NF-κB binding affinity and specificity.

Genetic and biochemical experiments using the homologous *Drosophila* Dorsal/Cactus system identified two Dorsal residues (Cys-233 and Ser-234) required for Cactus binding (Lehming et al., 1995). The presence of similar residues in p65 (Asn-202 and Ser-203), but not p50 (*Thr-256* and *Ala-257*), invoked the possibility that these residues were involved in discriminatory interactions between IκBα and NF-κB dimers. As the IκBα/NF-κB complex structure shows, however, IκBα contacts the face of p65 opposite the sites of the Dorsal mutations and interacts with the corresponding p50 residues. Furthermore, immunoprecipitation experiments show that IκBα interacts with comparable stability with homodimers of p50 and p65 dimerization domains (Latimer et al., 1998). Therefore, although the dimerization domain mediates extensive contacts and contributes significantly to overall binding affinity, this region does not appear to be responsible for conferring differential binding specificity upon IκBα.

In all likelihood it is the net contribution of several interactions between the amino-terminal domain of p65 and the incomplete sixth ankyrin repeat of IκBα that dictates NF-κB p50/p65 heterodimer binding specificity. Among the proposed specificity-determining amino acids are Glu-49, Arg-50, and His-181 of p65 (Figure 5a). The corresponding residues in p50, *Ser-71*, *Asp-235*, and *Ala-70*, cannot mediate similar interactions with Trp-258, Gln-266, and Met-279 of IκBα. By positioning its side chain deep into the cavity created at the junction of the p50 dimerization domain, the p65 amino-terminal domain, and the ARD of IκBα, Arg-50 contacts multiple acidic residues. His-181 stacks against Trp-258 of IκBα and makes van der Waals contacts with Met-279 of IκBα. The carboxylate group of Glu-49 receives one hydrogen bond from a backbone nitrogen of Gly-259 in IκBα. Together these residues may contribute significantly to the total binding free energy.

Conclusion

The IκBα/NF-κB complex crystal structure shows how the IκBα molecule recognizes and inactivates the NF-κB p50/p65 heterodimer. An extensive binding interface mediates numerous specific intermolecular contacts involving each of the structural segments of NF-κB and IκBα. The structure reveals the DNA inhibitory binding conformation of NF-κB. Adoption of this closed conformation is afforded by the flexible linker connecting the amino-terminal and dimerization domains of p65 and stabilized through interactions with the IκBα molecule. IκBα/NF-κB complex formation results in cytoplasmic retention of both complex components. This results from the association of ankyrin repeats 1 and 2 of IκBα with the NLS polypeptides of NF-κB and is, perhaps, aided by adoption of the closed conformation of NF-κB.

Experimental Procedures

Protein Expression, Purification, and Complex Formation

An IκBα expression plasmid was prepared by polymerase chain reaction (PCR) amplification of the region of MAD-3 cDNA encoding IκBα residues 67–302 and subsequent ligation into the NdeI and BamHI sites of pET 11a (Novagen). In a similar fashion, p50 dimerization domain and p65 rel homology region DNA constructs were ligated sequentially into a double expression vector prepared in this laboratory (F. Chen, submitted). Proteins were expressed in *Escherichia coli* strain BL21 (DE3). IκBα bacterial lysates were purified on Q sepharose (Pharmacia) and hydroxyapatite Bio-Gel (Bio-Rad) columns. After DNA precipitation with streptomycin sulfate, the NF-κB bacterial lysates were purified on SP sepharose (Pharmacia) columns. Both proteins were then purified independently by size exclusion chromatography on a SuperDex 75 gel filtration column (Pharmacia). The purified components were next combined with a 1.2-fold molar excess of IκBα. The IκBα/NF-κB complex was purified by a second round of Superdex 75 size exclusion column chromatography. Finally, the purified complex was concentrated to 35 mg/ml by centricon-30 (Amicon).

Crystallization and Data Collection

Crystals containing the IκBα/NF-κB complex were grown by the hanging drop vapor diffusion method. Drops containing 4 μl of protein (7 mg/ml) in 25 mM MES buffer (pH 6.35), 6.5% polyethylene glycol (PEG) 8000, and 2.5 mM dithiothreitol (DTT) were equilibrated against 1 ml of reservoir solution containing 50 mM MES (pH 6.35), 10% PEG 8000, and 5 mM DTT at 23°C–24°C. Rod-like crystals (0.5 × 0.1 × 0.05 mm) formed in 2–3 days amid heavy precipitate. Initial characterization of these crystals indicated that they belong to the monoclinic space group C2 with unit cell dimensions a = 124.5 Å, b = 49.3 Å, c = 120.6 Å, and β = 108.7°. The IκBα/NF-κB complex cocrystals contain one complex per asymmetric unit and are 43% solvent by volume. Crystals were introduced into cryo-protectant solution containing 50 mM MES (pH 6.35), 10% PEG 8000, 30% glycerol and flash cooled in liquid nitrogen. The crystals diffract to 3.1 Å with laboratory source X-rays and to at least 2.3 Å with a synchrotron radiation source. Home source data were taken at 105 K using CuKα radiation produced by a Rigaku rotating anode FR5 X-ray generator equipped with Charles Supper focusing mirrors and measured with a MAR research image-plate detector. Data taken at the Brookhaven NSLS beamline X25 were collected using a Brandeis 2 × 2 CCD detector, and X4A data were measured with an R-Axis detector. All data processing was with the DENZO/HKL package (Otwinowski, 1993).

Structure Determination and Refinement

An NF-κB p50/p65/DNA complex structure dimerization domain model was used to locate the position of the identical dimerization domains in the IκBα/NF-κB complex. AMoRe (Navaza, 1994) rotation and translation functions revealed a solution using data from 8 to 4 Å from the Native 1 data set. To locate the amino-terminal domain of p65 in the IκBα/NF-κB complex, a model of this domain from the DNA-bound heterodimer structure was employed. No clear translation solution was evident. With the dimerization domains fixed, the best 50 rotation solutions were used to find the proper translation solution. The seventh rotation solution against Native 1 data at 7 to 3.5 Å gave a translation solution with correlation coefficient of 25.5.

Molecular replacement phases calculated from NF-κB were not sufficient to determine the complex structure. To obtain additional phase information, we carried out multiple isomorphous replacement (MIR) with two heavy metal derivatives: 1 mM phenylmercuric acetate (soaked for 6 hr) and 0.5 mM $K_2[PtCl_4]$ (soaked for 3 hr). Four Hg atoms were identified by difference Patterson analysis using the program PHASES (Furey and Swaminathan, 1990). The other two Hg sites and all platinum sites were identified by difference Fourier synthesis. The phasing parameters were refined in MLPHARE CCP4 (CCP4, 1994). The MR and MIR phases were combined at 3.1 Å resolution, and the combined phases were improved by histogram matching and solvent flattening with the program DEPHASE (Greg Van Duyne, personal communication). The figure of merit after phase combination was 0.45 (acentric) and 0.53 (centric).

Interpretable density for the entire complex was observed except for amino- and carboxy-terminal regions of IκBα, the NLS polypeptides, and some loops of the complex. Using known ARD-containing structures as a guide, the IκBα ARD was fitted to the map. Other missing portions were gradually incorporated into the model by calculating $|2F_o\text{-}F_c|$ maps.

Model building utilized the program TOM/FRODO (Cambillau and Horjales, 1987) and O (Jones et al., 1991). Structure refinement was done with X-PLOR (Brünger, 1992) and then CNS (Brünger et al., 1998) using both conjugate gradient minimization and simulated annealing. The current model includes 112 of the 118 amino acid residues from p50 (the last six amino acids are not included), 279 of 285 residues from p65 (residues 189 and 190 from the flexible linker and the last four amino acids are not included), 216 out of 235 from IκBα (the amino-terminal four, four from the loop of ankyrin repeat 1, and the carboxy-terminal 11 amino acids are not included), and 214 water molecules. Only two of 607 residues, Phe-106 and Leu-131 of IκBα, fall in disallowed regions of the Ramachandran plot, and 77% are in most favored regions. The average temperature factors of IκBα, p50 dimerization domain, p65 dimerization domain, p65 amino-terminal domain, and 214 water molecules are 42, 36, 37, 51, and 44 $Å^2$, respectively. Structures were analyzed and displayed using PROCHECK (Laskowski et al., 1993), SETOR (Evans, 1993), and GRASP (Nicholls, 1992) programs.

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References

Arenzana-Seisdedos, F., Thompson, J., Rodriguez, M.S., Bachelerie, F., Thomas, D., and Hay, R.T. (1995). Inducible nuclear expression of newly synthesized IκBα negatively regulates DNA-binding and transcriptional activities of NF-κB. Mol. Cell. Biol. *15*, 2689–2696.

Arenzana-Seisdedos, F., Turpin, P., Rodriguez, M., Thomas, D., Hay, R.T., Virelizier, J.L., and Dargemont, C. (1997). Nuclear localization of IκBα promotes active transport of NF-κB from the nucleus to the cytoplasm. J. Cell. Sci. *110*, 369–378.

Baeuerle, P.A., and Baltimore, D. (1988). IκB: a specific inhibitor of the NF-κB transcription factor. Science *242*, 540–546.

Baeuerle, P.A., and Baltimore, D. (1996). NF-κB: ten years after. Cell *87*, 13–20.

Baeuerle, P.A., and Henkel, T. (1994). Function and activation of NF-κB in the immune system. Annu. Rev. Immunol. *12*, 141–179.

Baldwin, A.S., Jr. (1996). The NF-κB and IκB proteins: new discoveries and insights. Annu. Rev. Immunol. *14*, 649–683.

Batchelor, A.H., Piper, D.E., de la Brousse, F.C., McKnight, S.L., and Wolberger, C. (1998). The structure of GABPα/β: an ETS domain-ankyrin repeat heterodimer bound to DNA. Science *279*, 1037–1041.

Beg, A.A., Ruben, S.M., Scheinman, R.I., Haskill, S., Rosen, C.A., and Baldwin, A.S., Jr. (1992). IκB interacts with the nuclear localization sequences of the subunits of NF-κB: a mechanism for cytoplasmic retention. Genes Dev. *6*, 1899–1913.

Bork, P. (1993). Hundreds of ankyrin-like repeats in functionally diverse proteins: mobile modules that cross phyla horizontally? Proteins *17*, 363–374.

Brown, K., Park, S., Kanno, T., Franzoso, G., and Siebenlist, U. (1993). Mutual regulation of the transcriptional activator NF-κB and its inhibitor IκBα. Proc. Natl. Acad. Sci. USA *90*, 2532–2536.

Brünger, A.T. (1992). X-PLOR, Version 3.1 Manual: A System for X-ray Crystallography and NMR (New Haven, CT: Yale Universty Press).

Brünger, A.T., Adams, P.D., Clore, G.M., Gros, P., Grosse-Kuntsleve, R.W., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, N.S., and Read, R.J. et al. (1998). Crystallographic and NMR system: a new software system for macromolecular structure determination. Acta Crystallogr. D, in press.

Cambillau, C., and Horjales, E. (1987). TOM: a FRODO subpackage for protein ligand fitting with interactive energy minimization. J. Mol. Graph. *5*, 174–177.

CCP4 (1994). The CCP4 suite: programs for protein crystallography. Acta Crystallogr. D *50*, 760–763.

Chen, Z., Hagler, J., Palombella, V.J., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T. (1995). Signal-induced site-specific phosphorylation targets IκBα to the ubiquitin-proteasome pathway. Genes Dev. *9*, 1586–1597.

Chen, F.E., Huang, D.-B., Chen, Y.-Q., and Ghosh, G. (1998a). Crystal structure of p50/p65 heterodimer of transcription factor NF-κB bound to DNA. Nature *391*, 410–413.

Chen, Y.-Q., Ghosh, S., and Ghosh, G. (1998b). A novel DNA recognition mode by the NF-κB p65 homodimer. Nat. Struct. Biol. *5*, 67–73.

Conti, E., Uy, M., Leighton, L., Blobel, G., and Kuriyan, J. (1998). Crystallographic analysis of the recognition of a nuclear localization signal by the nuclear import factor karyopherin α. Cell *94*, 193–204.

Cramer, P., Larson, C.J., Verdine, G.L., and Müller, C.W. (1997). Structure of the human NF-κB p52 homodimer-DNA complex at 2.1 Å resolution. EMBO J. *16*, 7078–7090.

de Martin, R., Vanhove, B., Cheng, Q., Hofer, E., Csizmadia, V., and Winkler, H. (1993). Cytokine-inducible expression in endothelial cells of an IκBα-like gene is regulated by NF-κB. EMBO J. *12*, 2773–2779.

Ernst, M.K., Dunn, L.L., and Rice, N.R. (1995). The PEST-like sequence of IκBα is responsible for inhibition of DNA binding but not for cytoplasmic retention of c-Rel or RelA homodimers. Mol. Cell. Biol. *15*, 872–882.

Evans, S.V. (1993). SETOR: hardware-lighted three-dimensional solid model representations of macromolecules. J. Appl. Crystallogr. *11*, 134–138.

Franzoso, G., Bours, V., Park, S., Tomita-Yamaguchi, M., Kelly, K., and Siebenlist, U. (1992). The candidate oncoprotein Bcl-3 is an antagonist of p50/NF-κB-mediated inhibition. Nature *359*, 339–342.

Furey, W., and Swaminathan, S. (1990). PHASES: a program package for the processing and anlaysis of diffraction data from macromolecules. Am. Crystallogr. Ass. Mtg. Summ. *18*, 73.

Ghosh, S., and Baltimore, D. (1990). Activation in vitro of NF-κB by phosphorylation of its inhibitor IκB. Nature *344*, 678–682.

Ghosh, G., Van Duyne, G., Ghosh, S., and Sigler, P.B. (1995). Structure of NF-κB p50 homodimer bound to a κB site. Nature *373*, 303–310.

Gorina, S., and Pavletich, N.P. (1996). Structure of the p53 tumor suppressor bound to the ankyrin and SH3 domains of 53BP2. Science *274*, 1001–1005.

Govind, S., Drier, E., Huang, L.H., and Steward, R. (1996). Regulated nuclear import of the Drosophila rel protein dorsal: structure-function analysis. Mol. Cell. Biol. *16*, 1103–1114.

Hatada, E.N., Nieters, A., Wulczyn, F.G., Naumann, M., Meyer, R., Nucifora, G., McKeithan, T.W., and Scheidereit, C. (1992). The ankyrin repeat domains of the NF-κB precursor p105 and the protooncogene bcl-3 act as specific inhibitors of NF-κB DNA binding. Proc. Natl. Acad. Sci. USA *89*, 2489–2493.

Henkel, T., Zabel, U., van Zee, K., Muller, J.M., Fanning, E., and

Baeuerle, P.A. (1992). Intramolecular masking of the nuclear location signal and dimerization domain in the precursor for the p50 NF-κB subunit. Cell *68*, 1121–1133.

Huang, D.-B., Huxford, T., and Ghosh, G. (1997). The role of DNA in the mechanism of NF-κB dimer formation: crystal structures of the dimerization domains of the p50 and p65 subunits. Structure *5*, 1427–1436.

Inoue, J.-I., Kerr, L.D., Rashid, D., Davis, N., Bose, H.R., and Verma, I.M. (1992). Direct association of pp40/IκBβ with rel/NF-κB transcription factors: role of ankyrin repeats in the inhibition of DNA binding activity. Proc. Natl. Acad. Sci. USA *89*, 4333–4337.

Jacobs, M.D., and Harrison, S.C. (1998). Structure of an IκBα/NF-κB complex. Cell, this issue, 749–758.

Jaffray, E., Wood, K.M., and Hay, R.T. (1995). Domain organization of IκBα and sites of interaction with NF-κB p65. Mol. Cell. Biol. *15*, 2166–2172.

Jones, T.A., Zou, J.-Y., Cowan, S.W., and Kjeldgaard, M. (1991). Improved methods for building protein models in electron density maps and the location of errors in these models. Acta Crystallogr. A *47* 110–119.

Laskowski, R.A., MacArthur, M.W., Moss, D.S., and Thornton, J.M. (1993). PROCHECK: a program to check the stereochemistry quality of protein structures. J. Appl. Crystallogr. *26*, 283–291.

Latimer, M., Ernst, M.K., Dunn, L.L., Drutskaya, M., and Rice, N.R. (1998). The N-terminal domain of IκBα masks the nuclear localization signal(s) of p50 and c-Rel homodimers. Mol. Cell. Biol. *18*, 2640–2649.

Lehming, N., McGuire, S., Brickman, J.M., and Ptashne, M. (1995). Interactions of a Rel protein with its inhibitor. Proc. Natl. Acad. Sci. USA *92*, 10242–10246.

Luh, F.Y., Archer, S.J., Domaille, P.J., Smith, B.O., Owen, D., Brotherton, D.H., Raine, A.R., Xu, X., Brizuela, L., Brenner, S.L., and Laue, E.D. (1997). Structure of the cyclin-dependent kinase inhibitor $p19^{INK4d}$. Nature *389*, 999–1003.

Malek, S., Huxford, T., and Ghosh, G. (1998). IκBα functions through direct contacts with the nuclear localization signals and the DNA binding sequences of NF-κB. J. Biol. Chem. *273*, 25427–25435.

McElhinny, J.A., Trushin, S.A., Bren, G.D., Chester, N., and Paya, C.V. (1996). Casein kinase II phosphorylates IκBα at S-283, S-289, S-293, and T-291 and is required for its degradation. Mol. Cell. Biol. *16*, 899–906.

Michaely, P., and Vennett, V. (1992). The ANK repeat: a ubiquitous motif involved in macromolecular recognition. Trends Cell. Biol. *2*, 127–130.

Müller, C.W., Rey, F.A., Sodeoka, M., Verdine, G.L., and Harrison, S.C. (1995). Structure of the NF-κB p50 homodimer bound to DNA. Nature *373*, 311–317.

Navaza, J. (1994). AMoRe: an auomated package for molecular replacement. Acta Crystallogr. A *50*, 157–163.

Nicholls, A. (1992). GRASP Manual (New York: Columbia University Press).

Otwinowski, Z. (1993). DENZO: An Oscillation Data Processing Program for Macromolecular Crstallogrpahy (New Haven, CT: Yale University Press).

Powell, P.P., Dixon, L.K., and Parkhouse, R.M.E. (1996). An IκB homolog encoded by african swine fever virus provides a novel mechanism for downregulation of proinflammatory cytokine responses in host macrophages. J. Virol. *70*, 8527–8533.

Revilla, Y., Callejo, M., Rodriguez, J.M., Culebras, E., Nogal, M.L., Salas, M.L., Vinuela, E., and Fresno, M. (1998). Inhibition of nuclear factor-κB activation by a virus-encoded IκB-like protein. J. Biol. Chem. *273*, 5405–5411.

Rogers, S., Wells, R., and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science *234*, 364–368.

Schwarz, E.M., Van Antwerp, D., and Verma, I.M. (1996). Constitutive phosphorylation of IκBα by casein kinase II occurs preferentially at serine 293: requirement for degradation of free IκBα. Mol. Cell. Biol. *16*, 3554–3559.

Siebenlist, U., Franzoso, G., and Brown, K. (1994). Structure, regulation and function of NF-κB. Annu. Rev. Cell. Biol. *10*, 405–455.

Stancovski, I., and Baltimore, D. (1997). NF-κB activation: the IκB kinase revealed? Cell *91*, 299–302.

Sun, S.-C., Ganchi, P.A., Ballard, D.W., and Greene, W.C. (1993). NF-κB controls expression of inhibitor IκBα: evidence for an inducible autoregulatory pathway. Science *259*, 1912–1915.

Sun, S., Elwood, J., and Greene, W.C. (1996). Both amino- and carboxyl-terminal sequences within IκBα regulate its inducible degradation. Mol. Cell. Biol. *16*, 1058–1065.

Thompson, J.E., Phillips, R.J., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1995). IκBβ regulates the persistent response in a biphasic activation of NF-κB. Cell *80*, 573–582.

Traenckner, E.B.-M., Wilk, S., and Baeuerle, P.A. (1994). A proteasome inhibitor prevents activation of NF-κB and stabilizes a newly phosphorylated form of IκBα that is still bound to NF-κB. EMBO J. *13*, 5433–5441.

Tran, K., Merika, M., and Thanos, D. (1997). Distinct functional properties of IκBα and IκBβ. Mol. Cell. Biol. *17*, 5386–5399.

Venkataramani, R., Swaminathan, K., and Marmorstein, R. (1998). Crystal structure of the CDK4/6 inhibitory protein $p18^{INK4c}$ provides insights into ankyrin-like repeat structure/function and tumor-derived $p16^{INK4}$ mutations. Nat. Struct. Biol. *5*, 74–81.

Verma, I.M., Stevenson, J.K., Schwarz, E.M., Van Antwerp, D., and Miyamoto, S. (1995). Rel/NF-κB/IκB family: intimate tales of association and dissociation. Genes Dev. *9*, 2723–2735.

Whiteside, S.T., Epinat, J.C., Rice, N.R., and Israël, A. (1997). IκBε, a novel member of the IκB family, controls RelA and cRel NF-κB activity. EMBO J. *16*, 1413–26.

Zabel, U., and Baeuerle, P.A. (1990). Purified human IκB can rapidly dissociate the complex of the NF-κB transcription factor with its cognate DNA. Cell *61*, 255–265.

Zabel, U., Henkel, T., Silva, M., and Baeuerle, P.A. (1993). Nuclear uptake control of NF-κB by MAD-3, an IκB protein present in the nucleus. EMBO J. *12*, 201–211.

Zhong, H., Voll, R.E., and Ghosh, S. (1998). Phosphorylation of NF-κB p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. Mol. Cell *1*, 661–671.

Brookhaven Protein Data Bank ID Code

Atomic coordinates for the IκBα/NF-κB complex have been submitted and assigned the ID code 1ikn.