Mechanism of IκBα Binding to NF-κB Dimers*

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X-ray crystal structures of the NF-κB-IκBα complex revealed an extensive and complex protein-protein interface involving independent structural elements present in both IκBα and NF-κB. In this study, we employ a gel electrophoretic mobility shift assay to assess and quantify the relative contributions of the observed interactions toward overall complex binding affinity. IκBα preferentially binds to the p50/p65 heterodimer and p65 homodimer, with binding to p50 homodimer being significantly weaker. Our results indicate that the nuclear localization sequence and the region C-terminal to it of the NF-κB p65 subunit is a major contributor to NF-κB-IκBα complex formation. Additionally, there are no contacts between the corresponding nuclear localization signal tetrapeptide of p50 and IκBα. A second set of interactions involving the acidic C-terminal/PEST-like region of IκBα and the NF-κB p65 subunit N-terminal domain also contributes binding energy toward formation of the complex. This interaction is highly dynamic and nonspecific in nature, as shown by oxidative cysteine cross-linking. Phosphorylation of the C-terminal/PEST-like region by casein kinase II further enhances binding.

The Rel/NF-κB family transcription factors perform a vital role in mediating the cellular response to stress, inflammation, the immune response, and apoptosis (1–4). Mammalian Rel/NF-κB family polypeptides include p65 (RelA), p50, p52, RelB, and the proto-oncoprotein c-Rel. These subunits associate in various combinations to form homodimers and heterodimers with distinct but overlapping functions. Among the most abundant and best understood of these dimers are the p50/p65 dimers independent of their DNA inactivation. IκBα inactivates the NF-κB p50/p65 heterodimer by masking the NF-κB nuclear localization sequences (6). Activation of signaling pathways by extracellular signals, such as tumor necrosis factor or interleukin-1, leads to proteolysis of IκBα, allowing active NF-κB p50/p65 heterodimer to translocate into the nucleus (7).

IκBα is composed of three distinct regions: an N-terminal signal receiving domain (SRD), a central ankyrin repeat-containing domain (ARD), and an acidic C-terminal/PEST-like region rich in the amino acids proline, glutamic acid, serine, and threonine (8). IκBα is phosphorylated multiple proteins in vivo. The C-terminal/PEST-like region in constitutively phosphorylated by casein kinase II (CKII) (9, 10), and the SRD contains sites of inducible, signaling-dependent phosphorylation (11). X-ray crystal structures of IκBα in complex with the NF-κB p50/p65 heterodimer reveal an extensive and complex protein-protein interface (12, 13). The structures reveal that an apparently flexible segment of the NF-κB p65 subunit, which contains the NLS, becomes ordered upon complex formation and makes several contacts with IκBα. Interestingly, no direct contact is observed between the corresponding segment of the NF-κB p50 subunit and IκBα. The structures also reveal that the N-terminal domain of the p65 subunit contacts the C-terminal/PEST-like region of IκBα and its own dimerization domain through long range electrostatic interactions. Included in this interaction are many of the amino acid residues that mediate NF-κB-DNA contacts. These electrostatic interactions and the accompanying conformational change observed in the NF-κB p65 subunit were proposed to provide the DNA-inhibitory binding function of IκBα.

Previous experiments have been carried out to define regions of NF-κB dimers that are important for IκBα binding (14). For these previous experiments, we made use of a fluorescence polarization competition assay to assess the roles of different deletion fragments of NF-κB and IκBα. However, this approach limited our studies to NF-κB deletion protein constructs that display appreciable DNA binding affinity. As a result, we were not able to assess the contribution to overall complex binding affinity by regions of the NF-κB dimers independent of their DNA binding sequences.

In this report we make use of a gel electrophoretic mobility shift assay to isolate and characterize the interactions observed in the NF-κB-IκBα x-ray crystal structures. Our results show that: 1) Escherichia coli expressed full-length IκBα binds the NF-κB p50/p65 heterodimer with the highest affinity of the three NF-κB dimers tested. IκBα binding affinity toward the p50 and p65 homodimers is 20- and 2.5-fold lower, respectively, than toward the p50/p65 heterodimer. 2) The segment C-terminal to the NF-κB p65 subunit NLS contributes 5- to 6-fold to the overall binding affinity both in the case of the p65 homodimer and the p50/p65 heterodimer. By contrast, the corresponding segment of p50 contributes only 2-fold to IκBα binding affinity. 3) Within the context of the NF-κB p50/p65

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The abbreviations used are: SRD, signal receiving domain; ARD, ankyrin repeat domain; CKII, casein kinase II; EMSA, electrophoretic mobility shift assay; NLS, nuclear localization signal; RS-IκBα, Sky1 phosphorylation site-tagged IκBα.
heterodimer, neither the N-terminal domain nor the NLS of p50 contributes to IxBα binding. In contrast, the corresponding segments of the p65 subunit do make significant contributions to overall binding affinity. 4) When combined with its unphosphorylated form, casein kinase II-phosphorylated IxBα binds the p50/p65 heterodimer and the p65 homodimer with 33- and 9-fold higher binding affinity, respectively. 5) Finally, we report oxidative cross-linking data that illustrate the dynamic nature of the electrostatic interactions between the acidic C-terminal/PEST-like region of IxBα and NF-kB.

MATERIALS AND METHODS

Cloning and Purification of RS-tagged IxBα—The RS-IxBα clone was created by ligating together three components. The components included one IxBα construct corresponding to amino acid residues 1–317, 1–302, or 1–287, the RS tag, and the pET15b vector (Novagen). The N-terminal primer used for IxBα amplification by polymerase chain reaction was: 5'-TAGCTAGTGATCCTTACGCGGCGAGCCGCG-3'. The SalI restriction site is underlined. The C-terminal primers all contained a BamHI restriction site (underlined) and had the following sequences: 317, 5'-TGCGAGGATCCCTATACAGCGAGCTGCGAC-3'; 302, 5'-GAAGCTGAGGATCCCTAACGCTCTCTGTCTGAACTC-3'; and 287, 5'-AGCTGAGGATCCCTATACAGCGAGCTGCGAC-3'. The RS tag was generated by annealing the two oligonucleotides with the following sequences: 5'-TATGAGAGATGCTCTGGGAAAATCGACTACCAACCCAGCG-3' and 3'-AATCTCCTGACGGGCTTTTCAATGGTGTTGGCAGCT-5'. The resulting double-stranded DNA was phosphorylated on its 5'-hydroxyl groups with polynucleotide kinase (New England BioLabs) prior to ligation with the IxBα insert and vector. The amino acid sequence encoded for by the RS tag oligonucleotide is MDRAPRERPRTRLE. This represents the specific phosphorylation site for Sky1 (the splicing kinase in yeast) as derived from its substrate Npl3 (the phosphorylated serine is underlined) (15). The pET15b vector additionally encodes an N-terminal hexahistidine tag, which was used to purify the protein using a Ni2+ affinity resin (His-Bind, Novagen).

Phosphorylation of RS-IxBα—Phosphorylation of each of the RS-IxBα constructs (1–317, 1–302, and 1–287) was carried out using either the enzyme Sky1 (a gracious gift from Brad Nolen) alone or in combination with casein kinase II (CKII, New England Biolabs). In both cases the buffer used was 20 mM Tris-HCl (pH 7.5), 50 mM KCl, and 10 mM MgCl2. RS-IxBα was added to 50 μM in 100 mM cold ATP and incubated at 30 °C for an additional 30 min. Excess [32P]ATP was then removed, and the buffer was exchanged by concentration in a centricon-10 (Amicon) followed by a protein buffer exchange G-25 spin column (Amersham Pharmacia Biotech). Thereafter, 0.5 mg/ml of NF-kB and 0.25 mg/ml of IxBα proteins (approximately equal molar amounts) were incubated in PBS at room temperature for 30 min. Excess [32P]ATP was added prior to running in 10% SDS-polyacrylamide gels. Protein was visualized by Coomassie staining, and band shifts were verified by Western blots with antibodies toward both p65 and IxBα.

RESULTS

Experimental Design—We have shown previously via competition assay that within the context of the NF-kB p50/p65 heterodimer, the dimerization domain only of p50 as well as the N-terminal and dimerization domains, and the NLS of p65 participate in complex formation with IxBα. We have also shown that the IxBα NLS does not contribute to overall NF-kB binding affinity (14). The x-ray crystal structures of the IxBα complex in the complex with the NF-kB p50/p65 heterodimer support these biochemical findings. Furthermore, the crystal structures revealed a role for a sequence of 17 amino acids immediately N-terminal to the NF-kB p65 subunit NLS (12) as well as identifying the probable mode of interaction between the C-terminal/PEST-like region of IxBα and NF-kB (13).

To quantitate the relative contributions of each of these segments toward overall NF-kB-IxBα binding affinity, we employed a gel EMSA. EMSAs were performed by titrating increasing concentrations of NF-kB dimer protein constructs against a constant concentration of IxBα. For this assay, an IxBα fusion protein was prepared containing a peptide derived from the yeast protein Npl3, a natural substrate for Sky1 (15). The tag was placed at the IxBα N terminus, allowing for 32P labeling to occur at a location that does not interact with NF-kB. IxBα is a substrate for multiple kinases including CKII (Fig. 1). In the absence of the SR tag, however, Sky1 fails to transfer phosphate to IxBα (data not shown). The many NF-kB homodimer and heterodimer constructs employed in this study (Table 1) were engineered to address the contribution of each of the structural elements toward IxBα complex formation. Fig. 2 shows a schematic representation of the p50 and p65 NF-kB monomers and IxBα and defines the specific elements we targeted.
Residues C-terminal to the p65 NLS Are Essential for IκBα Binding—Crystal structures of the NF-κB-IκBα complexes have defined the segments of the p50 and p65 subunits that are involved in IκBα binding. The structure reveals that IκBα binds to the NF-κB p65 subunit within the region encompassing residues 19–319. The homologous segment in p50 includes residues 39–376 and corresponds to the entire p50 subunit through the NLS polypeptide. The crystal structures further reveal that, within the context of the NF-κB p50/p65 heterodimer, only the dimerization domain of p50 (residues 245–350) contacts IκBα. We have constructed and purified a slightly longer version of p65 ending at residue 325 for use in our binding experiments. We also constructed the homologous p50 homodimer and the p50/p65 heterodimer.

Table I

| NF-κB constructs used in EMSAs with RS-IκBα |
|---------------------------------------------|
| The following constructs were all used in EMSAs to determine their affinities for full length IκBα1–317. Additionally the full-length NF-κB heterodimers were used to determine the effects of C-terminal IκBα truncation. See Fig. 2 for a summary of the numbering of the NF-κB monomers. |
| p50 homodimer | p65 homodimer | p50/p65 heterodimer |
| p5039–376 | p6519–325 | p50245–376/p6519–325 |
| p5039–363 | p6519–304 | p50245–376/p6519–325 |
| p5039–363/p6519–325 | p5039–363/p6519–304 |

Fig. 1. Sky1 and CKII phosphorylate RS-IκBα in vitro. SDS-polyacrylamide gel electrophoresis analysis and autoradiography confirms that the kinases used in this study are capable of phosphorylating each of the three RS-IκBα protein constructs (ending at residues 287, 302, and 317).

Fig. 2. Schematic representations of p50, p65, and IκBα. The Rel homology regions of p50 and p65 are represented schematically. The numbering corresponds to the boundaries of the various NF-κB constructs used. For p65, two different N termini (1 and 19) and C termini (321 and 325) are used interchangeably because they bear no effects on IκBα binding. IκBα contains three distinct regions the SRD, ARD, and C-terminal/PEST-like region. Arrows indicate the borders of the constructs tested. Also indicated is the portion of the PEST-like region containing the five CKII phosphorylation sites.

Fig. 3. Binding of full-length NF-κB to IκBα. Gel shift assays showing the binding profiles of the p5039–376 (A) and p6519–325 homodimers (B) and the corresponding p50/p65 heterodimer (C). NF-κB concentration is indicated above the gels. Arrows indicate the positions of the bound and free IκBα. The schematic diagrams to the right of each gel correspond with Fig. 2. Note that the binding of p50 is much weaker than p65 or p50/p65. Also visible is the intermediate band on the p65 gel that is highlighted by asterisks.
TABLE II
p50/p65 heterodimer binding to RS-IκBα1–317

The p50/p65 heterodimer binds more tightly to IκBα than both the corresponding p50 and p65 homodimers. $K_{d,\text{obs}}$ is the average observed dissociation from a minimum of three independent experiments. The reported errors represent one standard deviation from the average value.

| NF-κB construct | $K_{d,\text{obs}}$ (nM) |
|-----------------|-------------------|
| p50245–376/p6519–325 | 2.07 ± 0.3 |
| p50245–376/p6519–325 | 6.00 ± 1.8 |
| p50245–376/p6519–325 | 9.90 ± 3.5 |
| p50245–376/p6519–325 | 27.1 ± 2.8 |
| p50245–376/p6519–325 | 32.6 ± 16 |
| p50245–376/p6519–325 | 77.9 ± 29 |

TABLE III
p65 homodimer binding to RS-IκBα1–317

The p65 homodimer binds IκBα slightly weaker than the p50/p65 heterodimer. “No quantifiable shift” observed for the 191–304 dimer indicates that the bound versus the free IκBα could not be resolved by EMSA because of complex instability.

| NF-κB construct | $K_{d,\text{obs}}$ (nM) |
|-----------------|-------------------|
| p65191–325 | 5.83 ± 1.6 |
| p6519–325 | 16.3 ± 4.0 |
| p6519–304 | 91.4 ± 22 |
| p65191–304 | No quantifiable shift observed |

TABLE IV
p50 homodimer binding to RS-IκBα1–317

The p50 homodimer binds IκBα weaker than either of the other dimers tested.

| NF-κB construct | $K_{d,\text{obs}}$ (nM) |
|-----------------|-------------------|
| p50245–363 | 217.6 ± 39.4 |
| p50245–376 | 414.6 ± 128 |
| p50245–363 | 443.8 ± 79.1 |
| p50245–363 | No quantifiable shift observed |

p50/p65 heterodimer. The p50 homodimer binds IκBα weakly with an equilibrium dissociation constant of 218 nM.

The structure of the NF-κB-IκBα complex solved by Jacobs and Harrison (12) revealed that the C-terminal 17 residues of IκBα mediate several contacts with IκBα. To assess and quantify the importance of these interactions in complex formation, we constructed a p65 homodimer ending at residue 304. The analogous construct of p50, corresponding to amino acids 39–363, was prepared as was the p50245–363/p65191–304 NF-κB heterodimer. EMSA results indicate that both the p50 homodimer and the p50/p65 heterodimer exhibit a 5–6-fold loss of binding affinity for IκBα upon removal of the segments C-terminal to the p50 NLS. By contrast, removal of this segment from the p50 homodimer results in a 2-fold loss in IκBα binding (Tables II–IV).

Role of the NF-κB N-terminal Domains in IκBα Binding—We tested for the role of the NF-κB N-terminal domains in binding IκBα. For this purpose, we constructed NF-κB p50/p65 heterodimers with one of the NF-κB N-terminal domains removed. The p50245–363/p6519–304 heterodimer binds IκBα with an affinity similar to that of p5039–363/p6519–304 heterodimer, suggesting that removal of the p50 N-terminal domain results in no change in overall binding (Table II). On the contrary, the p5039–363/p6519–304 heterodimer exhibited an approximately 3-fold decreased affinity toward IκBα, implicating a role for the C-terminal 17 residues of p50.

p65 subunit N-terminal domain in the complex interaction. These data correspond with the documented preference of IκBα toward the NF-κB p65 subunit (Fig. 4).

We measured affinities of the N-terminal truncated proteins in the context of their residues C-terminal to the NLS. Addition of p50 and p65 subunit C-terminal extensions produced little significant changes (Table II). Only a slight decrease in affinity was observed in the case of p50245–363/p6519–325, with the p65 N-terminal domain removed. Surprisingly, deletion of the p50 N-terminal domain resulted in 3–4-fold tighter binding for p50245–376/p6519–325 as compared with the full p5039–363/p6519–325 heterodimer. Similar results were obtained for the p65191–304 heterodimer with both the N-terminal domains removed. Specifically, the p65191–325 homodimer binds IκBα 2.5-fold tighter than does the homodimer p6519–325 (Table III). Removal of both of the N-terminal domains of the p50 homodimer causes a slight (2-fold) decrease in its affinity for IκBα (Table IV). We also tried to measure the IκBα binding affinity of p50 and p65 homodimers with both the N-terminal domains and the C-terminal extensions removed (p50245–363 and p65191–304). No quantifiable shift was observed, indicating that IκBα binds very poorly to these p50 and p65 homodimers (data not shown).

IκBα Binds One of the Two NLSs of NF-κB p50/p65 Heterodimer—Both x-ray NF-κB-IκBα complex crystal structures indicated that the NF-κB p50 subunit NLS does not contact IκBα (Fig. 5). To test whether this is indeed the case in solution, we created three different NF-κB heterodimers containing progressively shorter p50 subunit NLS polypeptide regions. The p65 NLS polypeptide remained unchanged in each case. These constructs were prepared in the context of the dimerization domains only of p50 and p65 subunits (starting at residues 245 and 191, respectively). Our results show that the presence of the NF-κB p50 subunit NLS in the p50245–363/p65191–325 does not enhance affinity of the heterodimer for IκBα when compared with the p50245–304/p65191–325 heterodimer that lacks the NLS (Table V). We do, however, observe a 2-fold increase in binding affinity for the p50245–376/p65191–325 heterodimer as compared with each of the other two, suggesting that amino acid residues C-terminal to the NLS of p50 may contact IκBα.

2 Phelps, C. B., Sengchanthalangsy, L. L., Malek, S., and Ghosh, G. (2000) J. Biol. Chem. 275, 24392–24399.
Interactions between IκBα and NF-κB

Fig. 5. The p50 NLS does not directly interact with IκBα. Representative gel shift assays for p50<sub>245–350</sub>/p65<sub>191–325</sub> (A), p50<sub>245–363</sub>/p65<sub>191–325</sub> (B), and p50<sub>246–376</sub>/p65<sub>191–325</sub> (C) heterodimers. Removal of the p50 NLS does not do effect binding, indicating that it does not contact IκBα. However, residues C-terminal to the NLS (364–376) are involved in contacting IκBα.

Table V
Role of the p50 NLS polypeptide

| NF-κB construct | K<sub>b</sub> |
|-----------------|------------|
|                 | nM         |
| p50<sub>245–376</sub>/p65<sub>191–325</sub> | 5.85 ± 0.74 |
| p50<sub>245–363</sub>/p65<sub>191–325</sub> | 11.1 ± 0.40 |
| p50<sub>246–376</sub>/p65<sub>191–325</sub> | 13.6 ± 3.5 |

without involving the p50 NLS itself.

Casein Kinase II Phosphorylation at the C-terminal/PEST-like Region of IκBα—The acidic C-terminal/PEST-like region of IκBα is constitutively phosphorylated in vitro by CKII (9, 10). This phosphorylation occurs on one or all of the three serines and two threonines that match the kinase consensus sequence. The x-ray crystal structures of the NF-κB/IκBα complex revealed that this region of IκBα is involved in contacting an extensive basically charged region of the p65 subunit N-terminal domain. We endeavored to understand what effects CKII phosphorylation of IκBα would have on complex formation. Using the same full-length IκBα construct as in the experiments previously described, EMSAs were performed with IκBα phosphorylated by both Syk1 and CKII. Our experiments show that IκBα phosphorylation by CKII can have a profound effect on NF-κB-IκBα complex formation. We tested both the p65<sub>1–325</sub> homodimer and the p50<sub>39–376</sub>/p65<sub>19–325</sub> heterodimer (Fig. 6). In both cases the affinity of the complexes was greatly enhanced, 9-fold stronger for p65 homodimer and 33-fold for the heterodimer (Table VI).

Further testing of the of the C-terminal/PEST-like region of IκBα in both its unphosphorylated and phosphorylated states revealed roles for this region in NF-κB binding. We observe that unphosphorylated IκBα truncated at its C terminus to either residue 287 or 302 exhibits similar NF-κB binding affinity as the full-length protein. We have shown previously that IκBα<sub>287–277</sub> fails to dissociate DNA-bound NF-κB as judged by our competition assay. Taken together, these data suggest that a minimal length IκBα required for binding and dissociation of DNA-bound NF-κB is IκBα<sub>287–277</sub>. Phosphorylation of IκBα<sub>287–277</sub> by CKII does not enhance affinity toward NF-κB p50/p65 heterodimers, and only marginal enhancement is observed in IκBα<sub>29–302</sub> upon CKII phosphorylation (Table VI). On the other hand, the affinity of full-length IκBα toward NF-κB increases dramatically after phosphorylation with CKII.

The C-terminal/PEST-like Region of IκBα Participates in Dynamic Interactions with NF-κB—The NF-κB/IκBα complex x-ray crystal structures reveal that the NF-κB p65 subunit adopts a conformation profoundly altered from that exhibited in its DNA-bound complex. The exact position of the N-terminal domain differs slightly in the two NF-κB/IκBα complex structures. Furthermore, the average temperature (B) factors of these p65 N-terminal domains are high. Both these observations suggest that the N-terminal domain of the NF-κB p65 subunit may participate with the IκBα PEST-like region in a dynamic interaction driven primarily through long range electrostatic contacts. Although intrigued that this significant change in NF-κB conformation could account for the DNA inhibitory binding activity observed upon binding to IκBα, the authors were careful to note that such an altered protein structure may, in fact, represent a crystal-packing artifact (12, 13).

In an effort to test whether the acidic C-terminal/PEST-like region of IκBα is, in fact, capable of such dynamic interactions with NF-κB, we performed the following oxidative cross-linking experiment. A mutant IκBα protein was prepared with serine-288, a side chain located within the C-terminal/PEST-like region, converted to a cysteine. This IκBα<sub>S288C</sub> protein containing the donor cysteine was incubated with three p65 homodimers, p65<sub>19–325</sub>/E22C, p65<sub>5–9–325</sub>/K221C, and p65<sub>19–325</sub>/R246C, each containing acceptor cysteine mutations at amino acid positions 22, 221, and 246, respectively. X-ray models
reveal that Arg^246 and Lys^221 are located 11 Å apart from each other on the bottom of the p65 dimerization domain. Glu^222 is positioned more than 20 Å from both Arg^246 and Lys^221 in the N-terminal domain of p65. We observe that IκBα binding affinity is increased by cross-linking of p65^67–302 S288C also cross-links, albeit with significantly lower efficiency, with the p65^67–288 S288C homodimer (Fig. 7).

**DISCUSSION**

The protein-protein interactions at the interface between the NF-κB p50/p65 heterodimer and IκBα are complicated in nature. This complexity arises from the involvement of multiple modular and flexible components at the complex interface: namely, the ARD and C-terminal/PEST-like region of IκBα, and the N-terminal domains, dimerization domains, and the NLS polypeptides of the NF-κB dimers. We endeavored to quantify the contributions made by each of these segments toward complex formation. In addition, we attempted to elucidate how CKII phosphorylation of the C-terminal/PEST-like region of IκBα modulates its binding to NF-κB dimers. Finally, we investigated the nature of the interaction between the NF-κB p65 subunit N-terminal domain and the acidic C-terminal/PEST-like region of IκBα.

Our results demonstrate that the interactions between IκBα and NF-κB dimers can be divided into two classes. As the x-ray crystal structures of the NF-κB-IκBα complex would clearly indicate, the NLS polypeptide of p65 contributes significantly toward IκBα binding affinity. The primary sequence of the NF-κB p50 and p65 subunit differs significantly in this region which might explain, at least in part, the selectivity of IκBα toward p65. We have also shown that in the p50/p65 heterodimer, the p50 NLS polypeptide, but not the NLS itself (amino acid residues 360–363), contributes to IκBα binding affinity. This observation is particularly interesting in light of several recent reports indicating that the inactive NF-κB p50/p65 heterodimer in complex with IκBα shuttles continuously between the cytoplasm and nucleus (20, 21). It would seem that although nuclear exit of this complex is mediated by a leucine-rich nuclear exit sequence within the IκBα SRD (amino acids 44–55) (22), constitutive nuclear import could depend on the partially unmasked p50 NLS.

Our experiments further demonstrate a second class of NF-κB-IκBα interactions involving the acidic/PEST-like region of IκBα and the NF-κB p65 subunit N-terminal domain. We show that although these contacts contribute to a lesser extent toward overall NF-κB-IκBα complex binding affinity, they probably affect significant functional consequences. We characterized these interactions as dynamic in nature. Our cross-linking experiments show that the cysteine mutant IκBα C-terminal/PEST-like region forms covalent bonds with a distance-dependent efficiency at three different positions on the p65 subunit covering a total distance of greater than 20 Å.

We observe that the N-terminally truncated p50/p65 heterodimer or p65 homodimer bind IκBα with higher affinities than the respective full-length dimers. This suggests that the absence of the N-terminal domains allows the acidic/PEST-like region of IκBα more freedom of movement, enhancing its ability to mediate further nonspecific interactions. Finally, we also tested which residues at the C terminus of IκBα are essential for NF-κB binding. We observe that in the absence of C-terminal phosphorylation by CKII the boundary is roughly residue Ser^287. However, this boundary changes when the acidic PEST is phosphorylated by CKII. It appears that the entire C terminus is required for full binding activity once the PEST-like region of IκBα is phosphorylated.

The NF-κB-IκBα x-ray crystal structures indicate that this second interaction that we have described juxtaposes two large, oppositely charged faces. One contains the acidic IκBα PEST-like region, and the other involves the basic DNA binding surfaces of the p65 N-terminal domain in a conformation radically different from that observed in its DNA-bound crystal structures. Though we still cannot rule out crystal packing interactions as a contributor to this observed "closed" conformation of the IκBα-bound NF-κB p65 subunit, these data support our proposed model of NF-κB DNA binding inhibition by electrostatic interference and long range, dynamic interactions with IκBα.

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**TABLE VI**

| IκBα construct | Phosphorylation | NF-κB | \(K_{d}^{\text{obs}}\) |
|----------------|----------------|-------|------------------|
| 1–317          | CKII/SKY1      | p50\_29–379/p65\_19–325 | 0.182 ± 0.040 |
| 1–317          | SKY1           | p50\_29–379/p65\_19–325 | 6.00 ± 1.8   |
| 1–317          | CKII/SKY1      | p65\_29–325             | 1.80 ± 0.68  |
| 1–317          | SKY1           | p65\_29–325             | 16.3 ± 4.0   |
| 1–302          | CKII/SKY1      | p50\_29–379/p65\_19–325 | 2.88 ± 0.54  |
| 1–302          | SKY1           | p50\_29–379/p65\_19–325 | 4.22 ± 0.59  |
| 1–287          | CKII/SKY1      | p50\_29–379/p65\_19–325 | 5.56 ± 0.60  |
| 1–287          | SKY1           | p50\_29–379/p65\_19–325 | 4.38 ± 1.0   |

FIG. 7. The C-terminal/PEST-like region of IκBα interacts dynamically with the dimerization and N-terminal domains of NF-κB. Nonreducing SDS-polyacrylamide gel electrophoresis analysis of oxidative cysteine cross-linking studies of the interaction of IκBα with p65 homodimer. Lanes are labeled by the constituents of the individual cross-linking reactions. Controls using p65^67–288 and IκBα^67–302 were run alongside the reactions of the cysteine mutants of p65 and IκBα^67–288 S288C. The IκBα^67–288 S288C protein runs at a higher molecular mass than the IκBα^67–302 because it contains a hexahistidine tag. The arrow indicates the location of the cross-linked p65/ IκBα complex.
Interactions between IκBα and NF-κB

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