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The RelA nuclear localization signal folds upon binding to IκBα

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Summary

The nuclear localization signal (NLS) polypeptide of RelA, the canonical NFκB family member, is responsible for regulating the nuclear localization of RelA-containing NFκB dimers. The RelA NLS polypeptide also plays a crucial role in mediating the high affinity and specificity of the interaction of RelA-containing dimers with the inhibitor IκBα, forming two helical motifs according to the published X-ray crystal structure. In order to define the nature of the interaction between the RelA NLS and IκBα under solution conditions, we conducted NMR and isothermal titration calorimetry (ITC) studies using a truncated form of IκBα containing residues 67-206 and a peptide spanning residues 293-321 of RelA. The NLS peptide, though largely unfolded, has a weak tendency toward helical structure when free in solution. Upon addition of the labeled peptide to unlabeled IκBα, the resonance dispersion in the NMR spectrum is significantly greater, providing definitive evidence that the RelA NLS polypeptide folds upon binding IκBα. ITC studies of single-point mutants reveal that residue F309, which is located in the middle of the more C-terminal of the two helices (helix 4) in the IκBα-bound RelA NLS polypeptide, is critical for the binding of the RelA NLS polypeptide to IκBα. These results help to explain the role of helix 4 in mediating the high affinity of RelA for IκBα.

Introduction

The nuclear factor kappa B (NFκB) family of transcription factors is a key component in the control of many cellular signaling events including cellular stress responses, cell growth, survival and apoptosis.^{1,2,3} The NFκB signaling system is activated by at least 58 viral or bacterial products, 46 stress conditions and chemicals, at least 32 cytokines and receptor ligands, as well as apoptotic mediators and mitogens, resulting in the transcription of more than 150 genes.⁴ In resting cells, NFκB dimers are sequestered in the cytoplasm via their interaction with proteins from the inhibitor of kappa B (IκB) family.⁵ IκBα, the most abundant and best-characterized member of the IκB family, is capable of inhibiting many of the NFκB family members, including the most abundant, NFκB(RelA (also known as p65)/p50), and other RelA and cRel-containing homo- and heterodimers.⁶ The NFκB-IκBα

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complex is highly stable in resting cells, with a half-life of >12 hours.⁷ However, even a small amount of NF κ B, which may be released due to leaky inhibition, would be sufficient to give gene expression; thus, a second tier of control, tight regulation of NF κ B nuclear localization, is employed to ensure cell homeostasis.^{7,8}

Regulation of nuclear localization of RelA occurs via its nuclear localization sequence (NLS), which targets it to the nucleus. Formation of the complex with I κ B α sequesters RelA-containing NF κ B in the cytoplasm by masking the RelA NLS within the binding interface.^{9,10} Upon stimulation, I κ B α is phosphorylated, ubiquitinated, and degraded, unmasking the RelA NLS and thus allowing its translocation to the nucleus and subsequent transcriptional activation activity. NLS sequences are generally short 3-20 amino acid sequences with a consensus sequence K-(K/R)-X-(K/R) that mediate nuclear import via recognition by the nuclear import receptor.^{11,12,13,14,15} The sequence KRKR, found at residues 301-304 within the RelA NLS polypeptide (generally identified as residues 289-321), is located at the carboxy-terminus of the rel homology domain of RelA (Figure 1A).¹⁶

The crystal structures of NF κ B (RelA/p50) bound to I κ B α reveal an extended protein-protein interface formed between I κ B α and NF κ B.^{10,17} I κ B α contacts NF κ B via its six ankyrin repeat domain and C-terminal PEST region, forming multiple contacts with the RelA NLS polypeptide, p50 and RelA dimerization domain and amino terminal domain regions that comprise NF κ B(RelA/p50).^{10,17} The two proteins run antiparallel, with ankyrin repeats 1-3 (AR1-3) contacting the RelA NLS polypeptide, which adopts a helix-turn-helix fold (Figure 1B).¹⁰ AR1 is capped by helix 4 of the RelA NLS polypeptide (residues 305-321) via hydrophobic contacts while AR1-AR3 contact helix 3 (residues 289-300), via mainly electrostatic contacts (Figure 1C).¹⁰ Meanwhile, AR3-6 and the first part of the I κ B α PEST sequence contact the NF κ B p50 and RelA dimerization domains and RelA amino-terminal domain.^{10,17} In contrast to the observed electron density for the RelA NLS polypeptide when bound to I κ B α , no density is observed for this region of NF κ B(RelA/p50) bound to DNA, suggesting that the RelA NLS is disordered and available for importin α binding until the NF κ B binds to I κ B α .^{10,18} In addition, The apparent disorder of the free RelA NLS suggests inherent flexibility that may facilitate adoption of different local structures in order to bind multiple targets, such as I κ B α and Importin α .¹⁹ In fact, NLSs bound to importin α usually adopt extended structures, while the RelA NLS polypeptide adopts a split helical conformation (termed helix3-helix4) when bound to I κ B α .^{10,20}

Thermodynamic and kinetic studies of the NF κ B(RelA/p50)-I κ B α interaction revealed that the interaction has an extremely low dissociation rate and a concomitantly high affinity, consistent with the long lifetime of the complex *in vivo*.²¹ This study also showed that the NF κ B(RelA/p50)-I κ B α interaction depends critically upon the RelA NLS, since truncating helix 4 of RelA resulted in a more than 1000-fold increase in the dissociation rate and 10,000-fold decrease in the affinity of I κ B α for NF κ B(RelA/p50) and contributed more significantly to the $\Delta C_{p,obs}$ and ΔG_{obs} than one would expect from such a small deletion.²¹ In the present study, we have employed NMR and isothermal titration calorimetry (ITC) in order to characterize the structural and dynamic changes in the RelA NLS polypeptide in the free and I κ B α -bound states in solution. The NMR studies presented here show that the RelA-NLS is disordered in the free state, and folds upon binding to I κ B α . In addition, mutagenesis reveals a single residue is critically important for high affinity.

Results

The minimal NLS and NLS-IkB α complex

Residues 289-321 of RelA are traditionally identified as the NLS peptide, although only the KRKR (residues 301-304) technically comprise the NLS. HSQC spectra of the peptide corresponding to RelA(289-321) showed missing peaks and uneven peak intensities that were localized to the flexible linker (residues 289-292) between the dimerization domain and helix 3 of the NLS. We therefore show only NMR spectra from the peptide corresponding to RelA(293-321), as it yielded a greater number of peaks of more even peak intensity in the complex even though the results presented here were obtained for both sequences. To form the minimal complex, a truncated form of IkB α , which contains only the first four ankyrin repeats of IkB α (residues 67-206),²² was used because in the crystal structure of the NF κ B-IkB α complex, the NLS polypeptide interacts only with the first three ankyrin repeats of IkB α . This minimal complex had significantly improved solubility characteristics for NMR study.²²

Backbone resonance assignments

The HSQC spectra of free and IkB α -bound RelA(293-321) are compared in Figure 2. Backbone resonance assignments of ^{15}N , ^{13}C -labeled RelA (293-321) free and in complex with IkB α (67-206) were obtained using standard triple resonance methods. Additional minor cross peaks were observed for residues 309-315, which we attribute to the possible presence of a small population of molecules containing an oxidized methionine at position 313. The HSQC spectra clearly show that in the free form the NLS polypeptide is unstructured, whereas a marked increase in resonance dispersion, consistent with the formation of a folded structure is observed for the IkB α -bound state (Figure 2).

Secondary structure of free and bound RelA NLS in solution

Secondary chemical shifts calculated from the difference between the observed and sequence-corrected random coil chemical shifts²³ are shown for ^{15}N , $^{13}\text{C}_\alpha$, $^{13}\text{C}_\beta$, ^{13}CO resonances of free and IkB α (67-206)-bound RelA(293-321) in Figure 3. Positive values of $^{13}\text{C}_\alpha$, $^{13}\text{C}_\alpha - ^{13}\text{C}_\beta$, and ^{13}CO and negative ^{15}N values show the presence of helical structure in bound RelA(293-321) (Figure 3B); residues 304-305, which are in a turn between helix 3 and helix 4 in the crystal structure of the IkB α -NF κ B complex show reduced secondary chemical shift values.¹⁰ Negative values of $^{13}\text{C}_\alpha$, $^{13}\text{C}_\alpha - ^{13}\text{C}_\beta$, and ^{13}CO were observed for the C-terminal residues 315-320, consistent with the lack of secondary structure seen in the crystal structure¹⁰ for this region.

The lower values of the secondary ^{15}N , $^{13}\text{C}_\alpha$, $^{13}\text{C}_\alpha - ^{13}\text{C}_\beta$, and ^{13}CO chemical shifts for free RelA NLS(293-321) (Figure 3A) were indicative of an unfolded peptide but it clearly has residual helical structure with helix 3 having a slightly greater helical propensity than helix 4. The slight helical tendency was also more pronounced in data collected at 278K.

Chemical shift perturbation

The differences in the chemical shifts between free and IkB α (67-206)-bound RelA NLS(293-321) are shown in Figure 4. The largest changes correspond to residues located in the turn between helix 3 and helix 4 and at the C-terminal ends of helix 3 (the NLS region) and helix 4 (residues 313-316). Large changes were also seen in Gly 320 for which multiple peaks were observed, possibly from conformational exchange due to its proximity to the C-terminus.

The resonances of IkB α (67-206) are also perturbed upon binding of RelA(293-321) (Figure 5). The most extreme chemical shift changes in IkB α (67-206) were observed for residues

that form contacts with the RelA NLS as seen in the crystal structure of the complex, indicating that binding of the isolated NLS polypeptide is the same as occurs when it is part of the RelA protein.

Backbone dynamics

Internal motions on a picosecond-nanosecond timescale were estimated by measuring the $^{15}\text{N}\{^1\text{H}\}$ heteronuclear NOE for free and IkB α (67-206)-bound RelA NLS(293-321). Heteronuclear NOE measurements were also collected for free RelA NLS (289-321) at 278K. The heteronuclear NOE values of the bound NLS ranged from 0.8 to 1, indicative of a well-structured peptide, except at the ends where values decrease below 0.5 (Figure 6A). Heteronuclear NOE values for RelA NLS(289-321) at 278K, are higher than those for RelA NLS(293-321) at 293K (open triangles) but lower than those seen for the complex. Slightly lower heteronuclear NOE values were measured for helix 4 of the complex at 278K in comparison to helix 3. The heteronuclear NOEs for the free RelA NLS(293-321) (open triangles), were below 0.7, characteristic of a disordered protein.

Hydrogen Exchange Probed by CLEANEX-PM Experiments

Further information on the conformational ensemble of free RelA NLS(293-321) was provided by amide exchange measurements made using the CLEANEX-PM method.²⁴ This experiment is useful for probing nascent structure in intrinsically disordered proteins.²⁵ In general, the intensity of the cross peaks increased with CLEANEX mixing time, indicating efficient exchange with the solvent, as expected for an unfolded peptide, but differences were observed between various parts of the sequence (Figure 6B). The bend between the two helices (residues 305- 307) showed the highest exchange rates. These residues also had lower heteronuclear NOEs (Figure 6A) and chemical shift values that were closer to random coil values (Figure 3A). Residues 302-304, 308-312 and 318 show lower CLEANEX ratios and higher heteronuclear NOEs, indicating relatively more nascent helical structure.

Alanine scanning mutagenesis identifies critical binding residues in the NLS

Previous experiments identified helix 4 as a hot spot for the NF κ B-IkB α interaction; this helix alone contributes some 7.8 kcal/mol to IkB α binding.²¹ To pinpoint the residues that contribute most to the binding affinity, the contact residues of the RelA NLS helix 4 identified from the crystal structure¹⁰ were each mutated to alanine. The results of these experiments are summarized in Table 1 and Figure 7. None of the mutations had a significant effect on the binding affinity of RelA(289-321) to IkB α (67-206) except for the F309A mutation, which dramatically decreased binding to an unmeasurable value (Figure 8B). In order to quantify the importance of F309 for binding in the context of NF- κ B as a whole, we introduced the F309A mutation in a construct co-expressing the heterodimer of the dimerization domains, RelA(190-321)/p50(248-350). Although the binding affinity of the wild type proteins are too high for accurate estimation of the K_D by ITC, the F309A RelA(190-321)/p50(248-350) bound at least 30-fold more weakly to IkB α (67-287) as indicated both by K_D and ΔH (Table 1).

Binding isotherms were also obtained at 50mM and 150 mM NaCl in order to probe the effect of electrostatic contacts, which appear from the crystal structure to mediate the binding of RelA NLS helix 3 to IkB α (Figure 1). The effect of ionic strength was fairly small, causing a less than two-fold difference in $K_{D,obs}$ for both RelA(293-321) and RelA(289-321) (Table 1).

We also measured the temperature dependence of the binding of RelA(289-321) to IkB α (67-287). The ΔH varied linearly with temperature resulting in a ΔC_p of -0.4 kcalM⁻¹K⁻¹ (Supp. Figure 1). Unfortunately, the ΔH decreased to zero at around 20°C, and

we were unable to measure it with confidence at lower temperatures to look for the curvature indicative of coupled folding and binding that has been observed by others.²⁶

Discussion

The RelA NLS folds upon binding I κ B α

I κ B α binds and retains NF κ B in the cytoplasm until the inhibition is relieved by degradation of I κ B α following receipt of an extracellular signal. Because no electron density was observed for RelA residues 289-321 in the DNA-bound NF κ B(RelA/p50) structure, the suggestion was made that the RelA NLS polypeptide might be unfolded in the absence of I κ B α .²⁷ Prediction of disorder propensity using the IUPred algorithm²⁸ also suggests that the NLS polypeptide of RelA (residues 289-321) should be disordered. The X-ray structure of the I κ B α -NF κ B complex that included residues 305-321 of RelA showed two different molecules in the asymmetric unit, one with the NLS polypeptide sandwiched between the top of I κ B α and the next molecule in the asymmetric unit, and the other with the NLS polypeptide only half-bound¹⁰. Our results using NMR experiments to elucidate the structure and backbone dynamics and ITC experiments to elucidate the thermodynamics of the I κ B α /RelA-NLS interaction provide the first direct experimental evidence that the NLS polypeptide folds upon binding I κ B α . The NMR data show that the free form is clearly in a disordered, highly dynamic configuration, although residual helical propensity in a few regions is definitely observed. The bound form adopts a stable helical conformation with much more well-dispersed signals indicative of a unique fully-folded and bound conformation. Reverse chemical shift perturbation allowed us to map the “footprint” of the NLS polypeptide onto the I κ B α and this “footprint” matches what would be expected from the crystallographically-determined structure in complex with I κ B α ¹⁰. To further confirm whether the NLS polypeptide was folding upon binding, we attempted to measure the temperature dependence of the ΔH observed during the binding reaction. A ΔC_p of $-0.4 \text{ kcalM}^{-1}\text{K}^{-1}$ was measured. However attempts to rationalize whether this was consistent with the expected surface area buried upon binding²⁹ were confounded by the fact that the NLS polypeptide was bound to differing extents in the two molecules in the asymmetric unit¹⁰, which yielded a range of predicted ΔC_p 's between -0.26 and $-0.41 \text{ kcalM}^{-1}\text{K}^{-1}$.

Mutagenesis identifies residues critical for the I κ B α -NF κ B interaction

The contributions to binding of I κ B α contacting residues within helix 4 of the NLS polypeptide, Tyr 306, Phe 309, Ile 312, Met 313, and Phe 318 was examined by ITC comparing the behavior of the wild type protein with mutants where these residues were substituted with alanine. Most of the mutations caused only slight decreases in binding affinity of the RelA NLS for I κ B α , but binding of the F309A mutant is virtually abolished. These studies therefore identify the hot spot³⁰ of this highly specific interaction as a single residue, Phe 309, which appears in the crystal structure¹⁰ to fit into a hydrophobic pocket on the top face of I κ B α formed by Phe 77 and Leu 80. This is in accordance with the prediction that Phe 309 would be crucial for binding of helix 4 to I κ B α , as it formed several native and non-native contacts with I κ B α in folding and binding simulations.³¹ In the present study Phe309 emerges as a sort of anchor helping to clamp the helix 4 to I κ B α .

The I312A mutant demonstrates enthalpy-entropy compensation: ΔH of binding increases by two-fold while $-T\Delta S$ decreases, thus achieving a similar binding affinity to that of the wild type peptide. Both the entropy decrease and enthalpy increase may be due to the substitution of a helix-disrupting β -branched side chain, which does not appear to be making any specific contacts in the bound structure, with alanine, which would promote helix formation. It is also interesting that mutation of Phe 318, which appears to contact I κ B α in the crystal structure¹⁰ (Figure 1C), had little effect on the binding affinity. This difference

may identify the area around Phe 318 as strongly influenced by crystal contacts into a conformation that is not characteristic of the complex in solution.

Significance of the NLS-IkBa interaction

The folding of the RelA NLS polypeptide upon binding to AR1-3 of IkBa explains the mechanism whereby IkBa-bound NFkB is retained in the cytoplasm. Although the crystal structure of the NFkB(RelA/p50)-IkBa complex hinted at the fact that the NLS was bound in a folded conformation on the surface of IkBa and therefore not accessible to Importin α , the crystallographic B-factors were extremely high, and the affinity of the binding was not known.¹⁰ Our results provide definitive evidence that the NLS polypeptide undergoes a disorder to order conformational change in the presence of IkBa, effectively masking it from binding by Importin α and inhibiting subsequent translocation to the nucleus. In addition, the helical structure induced by IkBa binding is incompatible with the extended structure required for Importin α interaction.

The tight binding affinity of the NFkB(RelA/p50)-IkBa complex is encoded in two interactions, one at either end of the large protein-protein interface.^{21,32} The interaction at the C-terminal part of IkBa involves the weakly-folded fifth and sixth ankyrin repeats of IkBa that fold upon binding the N-terminal part of NFkB.³³ We demonstrate that at the opposite end of the interface, the C-terminal NLS polypeptide of RelA also folds upon binding IkBa. Thus, the interaction may be said to follow a mutually synergistic “I-fold-you, you-fold-me” mechanism. Even though both helix 3 and helix 4 of the NLS polypeptide fold upon binding to IkBa, it is the last helix, helix 4, which is critical for the interaction.²¹ Indeed, a single residue within helix 4, Phe309, is all-important for the binding affinity. We may visualize the Phe309 ring acting as a “button”, fixing helix 4 to the “buttonhole” formed in the top face of the ARD of IkBa by Phe 77 and Leu 80. This highly specific interaction effectively caps the ankyrin repeat domain³⁴ generating significant favorable binding energy.

Materials and Methods

Protein expression and purification

The C-terminal sequences 289–321 and 293–321 of the RelA subunit of NFkB were expressed in the trp leader vector, which contains a His₈-tag and thrombin cleavage sequence, and drives small peptides into inclusion bodies.³⁵ Expression of unlabeled, [¹⁵N], [²H, ¹⁵N], [¹³C, ¹⁵N], and [²H, ¹³C, ¹⁵N]-labeled peptides was carried out in M9 minimal media in H₂O or D₂O supplemented with ¹⁵NH₄Cl (2 g/L) and ¹³C-glucose (8g/L) as needed. Cells were acclimated by growing them sequentially in 10 mL cultures of M9ZB, M9, M9(50% D₂O) and M9 (90% D₂O). Three one-liter growths were inoculated with the M9(90% D₂O) culture and induced at OD₆₀₀ = 1.0 with 0.5 mM IPTG for 12 hours at 37° C. The cells were collected by centrifugation at 5000 rpm for 30 minutes. Inclusion bodies were solubilized with 6M guanidine hydrochloride, 50 mM Tris (pH 7.5) and the solubilized peptide was captured by a Ni-NTA column equilibrated in the same buffer. A gradient was run to a final concentration of 150 mM NaCl, 50 mM Tris (pH 7.5), 2 mM CaCl₂. The peptide was cleaved from the column-bound fusion protein by incubating with thrombin (0.04 mg) in three column volumes by rocking the column with for 4 h at 25 °C three consecutive times. The final purification step was reverse-phase HPLC on a (C18 column (15 μ m, 300 $\times$ 19 mm I.D.) with a 0–50% acetonitrile gradient, with 0.1% (v/v) TFA. The peptide was lyophilized and dissolved in 25 mM Tris (pH 7.5), 0.5 mM EDTA, 50 mM NaCl and the pH adjusted with 10 M NaOH in 25mM Tris, 0.5 mM EDTA and 50 mM NaCl. The masses of all final purified peptides were verified by MALDI and ESI mass spectrometry. To study the binding of the NLS polypeptides to IkBa(67-206) by NMR, both

the NLS polypeptide and the I κ B α (67-206) were deuterated to minimize exchange broadening.

To monitor chemical shift perturbation of the I κ B α (67-206) chemical shifts upon titration with unlabeled NLS 289-321, ^{15}N -labeled I κ B α (67-206) was prepared as previously described.²² For NMR studies of the complexes of labeled NLS polypeptides with I κ B α (67-206), [^2H]-labeled I κ B α (67-206) was prepared in M9 minimal media in D_2O . Cells were acclimated by growing them sequentially in 10 mL cultures of M9ZB, M9, M9(50% D_2O) and M9 (90% D_2O). Two one-liter growths were inoculated with the M9(90% D_2O) culture and induced at $\text{OD}_{600}=0.6$ with 0.2 mM IPTG for 24 hours at 18° C. For all I κ B α (67-206) preparations, the cells were collected by centrifugation at 5000 rpm for 30 minutes and resuspended in 70 mL/liter of culture of 25 mM Tris (pH7.5), 50 mM NaCl, 0.5 mM EDTA, 10 mM β -mercaptoethanol, 0.3 mM PMSF, and protease inhibitor cocktail (Sigma) and lysed by sonication on ice. The soluble part of the lysate was purified by cation exchange chromatography on a Hi-Load Q-Sepharose 26/10 column (GE Healthcare) using a one hour gradient from 50 to 500 mM NaCl. Final purification of the protein was done on a HiLoad Superdex 75 16/60 gel filtration column (GE Healthcare). Final purification of the protein was done on a HiLoad Superdex 75 16/60 gel filtration column (GE Healthcare). The purified protein was concentrated in 4mL 10K MWCO Amicon concentrators (Millipore) in a fixed angle rotor at 4000 rpm for 15 minute intervals to prevent aggregation.

NMR Experiments and Data Analysis

The structural and dynamic properties of free and bound RelA NLS were studied by NMR. NMR experiments for sequence-specific resonance assignment RelA NLS(298-321) and (293-321) were carried out at 293 K to obtain sequence-specific assignment of H^{N} , N, C', C $^{\alpha}$, and C $^{\beta}$ resonances for free and I κ B α (67-206)-bound RelA NLS. A combination of HN(CA)CO, HNCO, and HNCACB were collected for the free RelA NLS(293-321) and HNCA, HNCB, HNCO and HN(CO)CACB for free RelA NLS(289-321) and bound RelA NLS (289-321) and (293-321).^{36,37,38} The details of how each experiment was performed are given in Supplementary Table 1. Spectra were processed with NMRpipe³⁹ and analyzed using NMRView.⁴⁰ Backbone resonance assignments of NLS constructs were determined by establishing connectivities using the HN(CA)CO and HNCO spectra and separately using HNCA, HNCB, and HN(CO)CACB in an iterative manner.

In order to probe the dynamics and structure of RelA NLS in its free and complexed forms, we also measured the $^{15}\text{N}\{^1\text{H}\}$ heteronuclear NOE of free and bound RelA NLS(293-321) at 293K. Saturated and unsaturated spectra were recorded in an interleaved manner. Hydrogen exchange in the of free and bound RelA NLS(293-321) at 293K was measured by fHSQC-CLEANEX-PM experiments.²⁴ To further characterize the structural and dynamic features of free RelA NLS(289-321), backbone resonance assignment experiments, $^{15}\text{N}\{^1\text{H}\}$ heteronuclear measurements and fHSQC-CLEANEX-PM experiments were also acquired at 278K in order to slow down conformational exchange of the free peptide.

Experimental details are reported in the Supporting Information (Supplementary Table 1). Data were processed using NMRpipe³⁹ and analyzed using NMRView⁴⁰ and Curvfit.⁴¹ The secondary structure propensity from the chemical shifts was determined by the approach described in,⁴² with random-coil reference chemical shift values^{43,44} corrected for primary sequence²³ and for deuterium isotope effects for the bound RelA NLS.⁴⁵

Isothermal Titration Calorimetry

ITC experiments were carried out on a Microcal VP ITC instrument. I κ B α (67-206) was purified by size exclusion chromatography (S75 column) immediately before use. In a

typical ITC experiment, 25 injections of 15 μ l of 1 mM RelA NLS or 0.05 mM NF κ B(RelA/p50) dimerization domains were made into either IkB α (67-206) (0.1 mM) or IkB α (67-287) (0.005 mM) in the cell. ITC experiments were carried out in a buffer of 25 mM Tris (pH 7.5), 0.5 mM EDTA, 50 mM NaCl, 1mM TCEP and 0.5 mM sodium azide at 293K in duplicate. Isotherms were analyzed using the Origin software (Microcal) as described.⁴⁶ The temperature dependence of binding of RelA-NLS(289-321) to IkB α (67-287) was measured in 150 mM NaCl, 10 mM MOPS, pH 7.5, and 0.5mM EDTA between 294 and 303K.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) The sequence of the RelA NLS polypeptide showing interactions with IκBα is shown below a schematic of the secondary structure of the RelA NLS from the x-ray crystal structure.¹⁰ Boxes indicate residues involved in interaction with IκBα: green, hydrophobic interaction; red and blue, electrostatic interaction. The residues of the putative NLS, KRKR are underlined and their position at the end of helix 3 indicated. (B) Ribbon diagram showing the x-ray crystal structure of the NFκB-IκBα complex.¹⁰ The p50 subunit is colored in green, the RelA DNA-binding and dimerization domains in red, and the RelA NLS polypeptide in magenta. IκBα is colored in blue. (C) Ribbon diagram showing the interactions between the RelA NLS polypeptide (magenta) and IκBα (grey). Residues making interactions are colored according to the boxes in part A.

Figure 2. Superposition of ^1H - ^{15}N HSQC spectrum of 0.2 mM ^1H - ^{15}N RelA-NLS(293-321)(black) with ^1H - ^{15}N HSQC spectrum of ^2H - ^{15}N RelA-NLS(293-321)(0.4mM)/ ^2H I κ B α (67-206) (0.6mM) (red), showing selected assignments. Both spectra were collected at 600MHz and 293K.

Figure 3.

Secondary chemical shifts of (A) free and (B) IκBα(67-206)-bound RelA-NLS. Cross peaks for residues 293-296 of the free peptide and 294-295 and 296-297 for the bound peptide were missing and these residues could not be assigned. Differences (in ppm) of observed *vs.* sequence-corrected random-coil values for Cα, Cα-Cβ, CO, and N chemical shifts are shown. A schematic of the secondary structure of the RelA-NLS from the x-ray crystal structure is shown at the top of each column.

Figure 4.

Chemical shift differences between the free and IkB α (67-206)-bound ^{15}N - RelA-NLS (293-321), calculated from chemical shifts measured in ^1H - ^{15}N HSQC spectra obtained at 800 MHz and 293 K according to the formula: $\Delta\delta=[(\Delta\delta_{\text{HN}})^2+(0.1\ \Delta\delta_{\text{N}})^2]^{1/2}$. A schematic of the secondary structure of the RelA-NLS from the x-ray crystal structure is shown at the top of the figure.

Figure 5.

Chemical shift differences between free and the RelA-NLS-bound ^{15}N - I κ B α (67-206) were calculated from chemical shifts measured in ^1H - ^{15}N HSQC spectra taken at 800 MHz and 298K and calculated in the same manner as in Figure 4. Inset: position of the amide N of residues with highest chemical shift differences between free and bound ^{15}N - I κ B α (67-206), are shown as spheres on the structure of I κ B α (67-206) from the NF κ B-I κ B α complex.

Figure 6.

(A) $^{15}\text{N}\{^1\text{H}\}$ Heteronuclear NOE values for free and IkB α (67-206)-bound RelA-NLS. Blue: ^2H - ^{15}N RelA-NLS(293-321)(0.4mM)/ ^2H IkB α (67-206)(0.6mM) [600MHz, 293K]; red: ^{15}N -RelA-NLS(289-321)(1 mM)[500 MHz, 278K]; green: ^{15}N p65(293-321) (0.3mM) [293K, 600MHz]. Error bars represent the deviation between two independent experiments. (B) Ratio of cross peak intensities between a CLEANEX-PM²⁴ spectrum recorded with zero mixing time and those at 2 msec (open circles) and 100 msec (filled circles). All spectra were collected on a 0.3mM ^{15}N RelA-NLS(293-321) sample at 501 MHz at 278K (red) and 293K (green).

Figure 7. ITC results for binding of (A) WT RelA-NLS(293-321) (B) F309A RelA-NLS(293-321). (C) WT RelA(190-321)/p50(248-350) (D) F309A RelA(190-321)/p50(248-350) to $\text{I}\kappa\text{B}\alpha(67-206)$ in 50 mM NaCl, 25 mM Tris, pH 7.5, 0.5mM EDTA, and 0.5mM sodium azide at 293K. Data were analyzed using a model for a single set of identical binding sites after the heat of dilution of NF κ B into buffer was subtracted. Thermodynamic values obtained from these curves are given in Table 1.

Table 1
Binding Thermodynamics of IκBα and p65 constructs measured by ITC

p65 NLS	Mutation	[NaCl] (mM)	K _D (nM)	ΔH (kcal mol ⁻¹)	-TΔS (kcal mol ⁻¹)	ΔG (kcal mol ⁻¹)
293-321	(WT)	50	371 ± 1	-4.2 ± 0.2	-4.4 ± 0.2	-8.6 ± 0.3
293-321	(WT)	150	746 ± 16	-4.3 ± 0.2	-3.9 ± 0.2	-8.2 ± 0.3
289-321	(WT)	50	800 ± 64	-3.7 ± 0.03	-4.4 ± 0.1	-8.1 ± 0.1
289-321	(WT)	150	1160 ± 13	-3.6 ± 0.4	-4.4 ± 0.4	-8.0 ± 0.8
289-321	Y306A	50	2600 ± 40	-2.3 ± 0.01	-5.2 ± 0.1	-7.5 ± 0.1
289-321	F309A	50	ND	ND	ND	ND
289-321	I312A	50	540 ± 2	-7.3 ± 0.2	-1.1 ± 0.01	-8.4 ± 0.02
289-321	M313A	50	970 ± 50	-3.0 ± 0.02	-5.1 ± 0.04	-8.1 ± 0.1
289-321	F318A	50	2700 ± 700	-2.5 ± 0.01	-5.0 ± 0.2	-7.5 ± 0.2
190-321	(WT)	50	<1	-12	ND	ND
190-321	F309A	50	19	-8	-2	-10

ND = Could not be determined