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Thermodynamics reveal that helix four in the NLS of NF- κ B p65 anchors I κ B α , forming a very stable complex

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SUMMARY

I κ B α is an ankyrin repeat protein that inhibits NF- κ B transcriptional activity by sequestering NF- κ B outside of the nucleus in resting cells. We have characterized the binding thermodynamics and kinetics of the I κ B α ankyrin repeat domain to NF- κ B(p50/p65) using surface plasmon (SPR) resonance and isothermal titration calorimetry (ITC). SPR data showed that the I κ B α and NF- κ B associate rapidly but dissociate very slowly, leading to an extremely stable complex with a $K_{D,obs}$ of approximately 40 pM at 37 °C. As reported previously, the amino-terminal/DNA binding domain of p65 contributes little to the overall binding affinity. Conversely, helix four of p65, which forms part of the nuclear localization sequence, was essential for high affinity binding. This was surprising given the small size of the binding interface formed by this part of the p65. The NF- κ B(p50/p65) heterodimer and p65 homodimer bound I κ B α with almost indistinguishable thermodynamics except that the NF- κ B p65 homodimer was characterized by a more favorable ΔH_{obs} relative to the NF- κ B (p50/p65) heterodimer. Both interactions were characterized by a large negative heat capacity change ($\Delta C_{p,obs}$), approximately half of which was contributed by the p65 helix four that was necessary for tight binding. This could not be readily accounted for by the small loss of buried non-polar surface area and we hypothesize that the observed effect is due to additional folding of some regions of the complex.

Keywords

Ankyrin repeat; NF- κ B; rel family; I κ B α ; surface plasmon resonance; isothermal titration calorimetry; transcription factor

INTRODUCTION

In resting cells, NF- κ B dimers with transcription activation potential are sequestered in the cytoplasm, interacting with a family of inhibitors of κ B (I κ Bs) proteins¹. The nuclear factor κ B (NF- κ B) is a family of transcription factors that control cellular signaling, cellular stress responses, cell growth, survival, and apoptosis²⁻⁵. It is known that at least 58 viral or bacterial products, some 46 stress conditions and chemicals, and at least 32 cytokines and receptor ligands, as well as apoptotic mediators and mitogens, activate the NF- κ B signaling system and subsequently the expression of more than 150 target genes⁶. I κ B α is capable of

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inhibiting many of the NF- κ B family members including the most abundant p50/p65 but also other p65 and cRel-containing homo- and heterodimers (for a review see ³). Following the action of a large number of different stimuli, I κ B α is phosphorylated, ubiquitinated, and degraded, freeing the NF- κ B nuclear localization signal (NLS) which targets the NF- κ B to the nucleus. In resting cells, the NF- κ B/I κ B α complex appears to be stable ⁷. A hallmark of the NF- κ B/I κ B α complex is its high stability in resting cells, with a recent estimate of the I κ B α half-life at longer than 48 hrs (Hoffmann et al., unpublished data). This tight regulation is critical, as only a small amount of NF- κ B, due to leaky inhibition, is sufficient to give gene expression. This is confirmed by a recent study which showed that upon stimulation, only a small fraction of cytoplasmic NF- κ B enters the nucleus and this results in transcriptional activation ⁸.

The crystal structure of NF- κ B(p50/p65) bound to I κ B α was determined by two labs simultaneously (Figure 1A) ^{9,10}. The structures reveal an extended protein-protein interface formed between I κ B α and NF- κ B, and provide a structural basis for the studies presented here. I κ B α contains an ankyrin repeat domain, comprising six ankyrin repeat units, and a C terminal PEST region. Each ankyrin repeat consists of about 33 residues and adopts a fold containing a β -hairpin fold followed by two anti-parallel α -helices followed by a short loop. The ankyrin repeat domain of I κ B α forms an extensive interface with the NF- κ B heterodimer, forming contacts in multiple regions (Figure 1B), burying more than 4000 Å² of surface area in the interface. The two proteins run antiparallel with ankyrin repeat 1 (AR1) of I κ B α being capped by helix four of NF- κ B p65 (residues 305-321). AR1 - AR3 contact helix three (residues 289-300) of p65, and AR3 - AR6 and the first part of the I κ B α PEST sequence contact the NF- κ B p50 and p65 dimerization domains. The PEST sequence also contacts the NF- κ B p65 amino-terminal domain. Despite the structural and biochemical information locating the contacting surfaces, identification of the critical determinants of binding affinity has eluded us ¹¹. In this study we address this important issue by taking apart the NF- κ B/I κ B α complex and carrying out a detailed study of the binding thermodynamics. We have previously investigated the interactions of the NF- κ B dimers with I κ B α by a fluorescence polarization competition assay ¹² and an electrophoretic mobility shift assay (EMSA) ¹³. However, both techniques have potential limitations and so we have undertaken a more detailed study by direct binding assays using surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC).

The binding thermodynamics and kinetics were measured for the interaction between I κ B α and full-length NF- κ B(p50/p65) heterodimer as well as with fragments of the NF- κ B that were missing either the N-terminal domain or helix four from the nuclear localization sequence (NLS) region. The interaction of I κ B α with the p65 homodimer was also measured. These experiments helped identify regions of NF- κ B that are critical for tight binding affinity and they also revealed the thermodynamics that drive the interaction.

We have previously demonstrated that the free I κ B α is highly dynamic in parts and only marginally stable in solution ¹⁴. In the work presented here, we also measured the temperature dependence of the binding interaction to investigate the possibility of a folding upon binding mechanism.

This study provides three important characteristics of the NF- κ B/I κ B α complex. First, the interaction has an extremely low dissociation rate which results in very high affinity and explains the long half-life observed *in vivo*. Second, the affinity of the interaction critically depends on the p65 NLS and this explains how the NLS is effectively sequestered by I κ B α . Third, the thermodynamics help explain how I κ B α can be very stable in complex with NF- κ B and yet be rapidly degraded.

RESULTS

I κ B α forms a very stable complex with NF- κ B

The binding kinetics and thermodynamics of the I κ B α /NF- κ B interaction were investigated by surface plasmon resonance (SPR). An N-terminal cysteine was introduced into NF- κ B(p65) and the NF- κ B(p50₂₄₈₋₃₇₆/p65₁₉₋₃₂₅) heterodimer was biotinylated and immobilized on a streptavidin chip. Sensorgrams reveal that the I κ B α /NF- κ B interaction achieved high affinity by a combined fast association rate and a slow dissociation rate at 37 °C (Figure 2A) leading to a $K_{D,obs}$ of $3.9 \pm 0.3 \times 10^{-11}$ M (Table 1A). Experiments were carried out at 37 °C to speed up the rate of dissociation of I κ B α , which was very slow at 25 °C, relative to the baseline drift of the instrument. Even at 37 °C the dissociation rate was very low, which meant that we did not even begin to cover one half-life of the dissociation event within the 20 min dissociation period. Thus, the dissociation rate we report represents the upper limit of the true k_d and the true $K_{D,obs}$ for the complex may be even lower. Disruption of the NF- κ B/I κ B α complex required a 1 min pulse of 3M urea at 37 °C and 6 M urea at 25 °C for regeneration. It is noteworthy that the NF- κ B(p50/p65) heterodimer was only tethered to the chip by the p65 domain, and the non-covalent interactions between the p50 and p65 were not disrupted during regeneration, suggesting that they are very strong. SPR binding data showed that two different I κ B α constructs comprising residues 67-287 or 67-317 bound to NF- κ B with almost identical kinetics and affinity ($K_{D,obs}$ of 3.9 ± 0.3 and $4.5 \pm 1.0 \times 10^{-11}$ M respectively) (Table 1A) demonstrating that the C-terminal residues of I κ B α , which extend the “PEST” sequence, do not have a significant effect on binding. Further experiments were performed using I κ B α ₆₇₋₂₈₇ because it was less prone to aggregation than the longer construct.

The NF- κ B p65 amino-terminal/DNA binding domain contributes minimally to I κ B α binding

The amino-terminal deletion construct, p65₁₉₀₋₃₂₁ (Figure 1B), was generated to investigate the effect of the p65 amino-terminal domain on I κ B α binding. The heterodimer was formed using an excess (1000x) of un-tagged p50 and sensorgrams were obtained as for the NF- κ B (p50₂₄₈₋₃₇₆/p65₁₉₋₃₂₅), except that only a 1 min pulse of only 1.5M urea was required for regeneration of the free NF- κ B on the chip (Figure 2B). Comparison of the results from NF- κ B (p50₂₄₈₋₃₇₆/p65₁₉₋₃₂₅), which contained the amino-terminal domain of p65 (Table 1A) with results for NF- κ B (p50₂₄₈₋₃₅₀/p65₁₉₀₋₃₂₁), lacking the amino-terminal domain revealed an approximately 2-fold decrease in k_a and an approximate 8-fold increase in k_d and a $K_{D,obs}$ of $3.2 \pm 1.0 \times 10^{-10}$ M (Table 1 B). The faster dissociation rate permitted data to be recorded at 25°C (Figure 2C) as well as 37°C (Table 1B) for this complex. As expected the dissociation rate was significantly slower at 25 °C resulting in a $K_{D,obs}$ of $0.91 \pm 0.27 \times 10^{-10}$ M.

Helix four of NF- κ B p65 plays a major role in the slow dissociation rate of the complex

The C-terminal residues of NF- κ B contain the NLS consensus sequence KRKR at residues 301 - 304. This region is flanked by two α -helices, helix three (289-300) and helix four (305-321). Together these motifs are known as the NLS domain (Figure 1B). We investigated the effect of helix four of the NLS on binding. Initial SPR experiments with NF- κ B (p50₂₄₈₋₃₅₀/p65₁₉₀₋₃₀₄) revealed that the I κ B α dissociated orders of magnitude faster; too fast to be quantitated at 37 °C. At 25 °C (Figure 3A) and at 15 °C (Figure 3C), the k_d of I κ B α was 100 ± 20 and $24 \pm 0.95 \times 10^{-3} \text{ s}^{-1}$ respectively, compared to $0.12 \pm 0.041 \times 10^{-3} \text{ s}^{-1}$ at 25 °C, for the longer construct (Table 1). Due to the weaker binding affinity, data were also analyzed by equilibrium analysis as shown in Figure 3B and 3D respectively. The results of both the kinetic and equilibrium analyses were in good agreement, confirming the reliability of both modes of analysis. Thus, deletion of helix four of the p65 NLS resulted in an increase of more than 1000 fold in k_d . Including the decrease in k_a , that was also observed, the affinity of I κ B α for this complex was decreased by approximately 10,000 fold by the deletion of helix four of the NLS. Although the NF- κ Bs that contained helix four bound too tightly to accurately

measure the $K_{D,obs}$ by ITC (Figure 2D), the NF- κ Bs that did not contain helix four bound weakly enough to allow direct comparison between the ITC and SPR data (Figure 4A, Table 2). The results showed good agreement between the two experiments, especially considering the complex nature of the two proteins involved. At 25 and 15 °C the $K_{D,obs}$ estimated by SPR was approximately four and three times weaker respectively than that measured by ITC. The small discrepancy probably results from an effect of the surface change of the carboxymethyl dextran chip or of the linker used to immobilize the NF- κ B. An electrostatic effect is more likely since the I κ B α contains an excess of acidic residues in its C-terminus. The ITC also confirmed the observation that the effect of the N-terminal domain on I κ B α binding affinity is small (Table 2, Figure 4B).

Since the NF- κ B NLS appeared so important for binding, we generated synthetic peptides spanning the regions of the p65 NLS. Three peptides comprising residues 289-307, 289-314 and 289-320 were synthesized. Only the longest peptide comprising residues 289-320, showed any detectable binding. An expressed version of this peptide that could be purified more readily bound with a $K_{D,obs}$ of $1.3 \pm 0.09 \times 10^{-6}$ M at 30 °C (Figure 5). The relatively tight binding affinity for such a short segment of NF- κ B underscores the importance of the NLS for binding to I κ B α . The observation that only peptides containing all of helix four bound I κ B α again highlights the importance of this region of the NLS.

Helix four of p65 contributes half of the observed large negative $\Delta C_{P,obs}$ for I κ B α /NF- κ B (p50p65) binding

Binding of NF- κ B to I κ B α was investigated over a range of temperatures using ITC. This technique provides a direct measure of the observed enthalpy change of binding (ΔH_{obs}) as well as the binding affinity. When carried out over a range of temperatures (at constant pressure), the observed heat capacity change ($\Delta C_{P,obs}$) and the full thermodynamic profile can be obtained. This was possible for the weaker binding I κ B α /NF- κ B(p50₂₄₈₋₃₅₀/p65₁₉₀₋₃₀₄) complex for which $K_{D,obs}$ and hence ΔG_{obs} and $T \cdot \Delta S_{obs}$ as well as the ΔH_{obs} could be accurately determined. The full thermodynamic profile for the interaction is shown in Figure 6A. The compensation of ΔH_{obs} and $T \cdot \Delta S_{obs}$ leading to the relatively small change in ΔG_{obs} over the physiological temperature range is apparent from this plot.

For the I κ B α /NF- κ B(p50₂₄₈₋₃₅₀/p65₁₉₀₋₃₂₁) complex containing the full NLS region, binding was too tight to permit determination of $K_{D,obs}$ directly. We were unable to employ a competition binding assay, due to the lack of a suitable competing ligand as used by Freire and co-workers^{15,16} and so only the ΔH_{obs} was determined for this interaction (Figure 6B). ΔH_{obs} was linear as a function of temperature up to approximately 33 °C, which is close to the temperature (45 °C) at which I κ B α unfolds to a soluble aggregate¹⁴. Above 33 °C the slope curved as also reported by Ladbury and co-workers, for another interacting system¹⁷. As discussed later on, only the linear region of the plot was analyzed yielding a $\Delta C_{P,obs} = -1.30 \pm 0.03$ kcal mol⁻¹ K⁻¹. This is a very large negative $\Delta C_{P,obs}$ even for such a large interaction interface suggesting additional contributions to the $\Delta C_{P,obs}$ that were not anticipated from the structure alone.

Comparison of the temperature dependence of the formation of the two I κ B α /NF- κ B complexes, one with and one without helix four in the NLS, revealed that deletion of helix four resulted in a $\Delta C_{P,obs}$ of only -0.60 ± 0.03 compared to -1.30 ± 0.03 when helix four was present. This was a surprisingly large difference in $\Delta C_{P,obs}$ for such a small deletion from the C-terminus of NF- κ B. Confirming these results, the $\Delta C_{P,obs}$ for the NLS peptide (residues 289-320) was measured to be -0.4 ± 0.04 (data not shown).

NF- κ B(p50p65) heterodimer and NF- κ B(p65p65) homodimer have an almost indistinguishable $K_{D,obs}$ and $\Delta C_{P,obs}$ for I κ B α binding

Binding of the NF- κ B(p65/p65) homodimer was measured by SPR to permit comparison with the heterodimer binding experiments. The NF- κ B(p65/p65) homodimer was immobilized on the chip by a single biotin linker on one of the p65 subunits. Analysis of the sensorgrams revealed binding characteristics almost indistinguishable from those for the NF- κ B(p50/p65) heterodimer (Table 1D). The association rates were similar (2.9 ± 0.3 compared to $1.7 \pm 0.32 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) as were the dissociation rates (1.6 ± 0.14 compared to $0.54 \pm 0.10 \times 10^{-3} \text{ s}^{-1}$), leading to less than 2 fold decrease in binding affinity for the p65 homodimer ($K_{D,obs} = 0.54 \pm 0.08 \times 10^{-9} \text{ M}$) relative to the heterodimer ($0.32 \pm 0.10 \times 10^{-9} \text{ M}$). This was also confirmed by the ITC data from the weaker binding complexes (Table 3). For example, $K_{D,obs}$ for the analogous heterodimer (p50₃₉₋₃₆₃/p65₁₋₃₀₄) and homodimer (p65₁₋₃₀₄/p65₁₋₃₀₄)/I κ B α complexes were 42 ± 6.0 and 51 ± 16 respectively. ITC data also showed that I κ B α binding to the NF- κ B(p65/p65) homodimer is characterized by a large negative $\Delta C_{P,obs}$ ($\Delta C_{P,obs} = -1.43 \pm 0.05 \text{ kcal mol}^{-1} \text{ K}^{-1}$) similar to that observed for the NF- κ B(p50/p65) heterodimer complex (Figure 7). Overall the binding of I κ B α to the homodimer is characterized by a more favorable (negative) ΔH_{obs} over the entire range of temperatures investigated, suggesting some compensation by a more unfavorable ΔS for the I κ B α /homodimer complex.

DISCUSSION

The I κ B α /NF- κ B complex is extremely high in affinity.

The NF- κ B signaling pathway is a complicated system involving the interactions of a large number of protein/protein and protein/DNA interactions¹⁸. In order to have a complete understanding of the mechanism of regulation in this system, to understand the subtle effects mutations play, and to perhaps build models that predict the system-wide behavior¹⁹, it is essential that the thermodynamics and kinetics of each of the interactions be accurately determined. We have used two direct binding experiments to measure the contributions of the individual domains of p65 to the I κ B α /NF- κ B interaction. When the entire RHR of p65 was present, the binding affinity was approximately 40 pM, some 25-fold tighter than previously reported¹³. Previous measurements of the I κ B α /NF- κ B binding affinity using a fluorescence polarization competition assay and by an electrophoretic mobility shift assay, reported values of approximately 1 nM^{9,13}. Both the ITC and the SPR data show that the affinity of NF- κ B heterodimers and homodimers containing full-length p65 is much tighter than the previously reported affinities. The agreement between the present data and previously reported data is much better for the weaker binding complexes, suggesting that the EMSA and fluorescence polarization assays were simply underestimating the high affinity of the complexes containing the entire RHR of p65. In resting cells, the I κ B α /NF- κ B complex is in the cytoplasm awaiting stimulation of the I κ B kinase (IKK) that phosphorylates I κ B α targeting it for ubiquitinylation and proteasome degradation³. Attempts to measure the intracellular half-life of the I κ B α /NF- κ B complex in resting cells report that the complex is exceedingly stable⁷. In IKK knock out cells, a half-life of at least 48 hours and perhaps longer has been estimated (A. Hoffmann, unpublished data). Our observations that the affinity of the I κ B α /NF- κ B complex is in the picomolar range, and that the dissociation rate constant is so slow as to be nearly irreversible, are consistent with the long half-life that has been observed *in vivo*. This is a very important finding because it helps to explain recent results showing that even upon activation, only a small fraction of the NF- κ B is actually released from its inhibited state to translocate into the nucleus and turn on transcription⁸. The extremely high affinity of the inhibited complex ensures that there is no "leakiness" in this transcriptional inactivation system, and allows for rapid transcriptional activation upon release of inhibition.

Helix four within the NLS is predicted to contain important specific contact residues.

We previously employed a structure-based mutagenesis approach to probe the interaction interface of the NF- κ B/I κ B α complex¹¹. This investigation showed that when more than 20 residues of I κ B α that form contacts with the NF- κ B were mutated to alanine only marginal effects on K_D were reported. This indicated a lack of a hot spot in the NF- κ B/I κ B α interface. Mutations that made the most significant effects were that of Tyr 181 to alanine, which makes contact to the p50 dimerization domain, and both Asp 71 and Asp 75 that make contact with Arg 304 located between helix three and helix four in the NF- κ B p65 NLS. When both residues are mutated to alanine an approximate 8-fold effect on binding of NF- κ B was observed¹¹. Based on the X-ray crystal structure, these residues seem to be important for anchoring down the NLS. This agrees with our finding that truncation of the NLS by deletion of helix four has a large effect on the K_D of the complex, contributing one third (4.5 kcal per mol) of the ΔG_{obs} at 25°C (Table 4). The total surface area buried by this region is only one sixth (818 Å² compared to 4902 Å²) of the total surface area buried by the whole RHR domain. The large contribution that this small region of p65 makes leads us to predict helix four within the p65 NLS (residues 305-320) will contain as yet unexplored specific contact residues important for the tight binding affinity of the NF- κ B/I κ B α interaction. This finding is biologically significant because tight binding of the NLS is expected to be important for sequestering the NLS from importin α . The KRKR sequence ending in Arg 304 forms the essential recognition motif for the importin complex²⁰⁻²² and a tightly bound helix four would most likely prevent specific interaction of the importin with the NLS.

The NF- κ B p65 homo dimer and NF- κ B(p50p65) heterodimer bind I κ B α equally well despite the difference in the dimerization domain interface.

Consistent with previous results, our equilibrium and kinetic binding experiments have revealed that the ΔG_{obs} and $\Delta C_{P,obs}$ as well as the k_a and k_d for the NF- κ B(p50p65) and the NF- κ B(p65p65) binding I κ B α are almost identical¹³. Given that there are significant differences in the specific contacting residues between the p50p65 and the p65p65 with I κ B α , there are two possible explanations for these results⁹. The residues that are non-conserved in p50 relative to p65 might be isoenergetic or enthalpy-entropy compensated in binding I κ B α . This possibility is supported by the significantly more favorable ΔH_{obs} for the NF- κ B(p65p65)/I κ B α complex, which is compensated by a more unfavorable ΔS contribution relative to the NF- κ B(p50p65)/I κ B α complex. However, in the absence of a crystal structure of the p65p65/I κ B α complex, it is difficult to rationalize this observation in detail. Another possible explanation is that the p65 subunit of the p50p65 contributes the major proportion of the ΔG_{obs} of binding and that the effect of subtle changes in contacting residues between the p50 subunit and the second p65 subunit in the homodimer/I κ B α is not significant. This possibility is supported by our finding that the NLS of p65 contributes almost half of the overall affinity of the complex of NF- κ B with I κ B α .

These biophysical data are important because they allow one to rationalize the biological effects of experiments, such as the p50 and p65 knock out experiments in mice. Mice lacking the p50 subunit of NF- κ B show no developmental abnormalities apparently because the p65 homodimer can replace all of the important functions of the normally more abundant p50p65 heterodimer²³. Conversely the p65 knock out is lethal in mice due to TNF- α induced apoptosis²⁴. In wild type cells, the NF- κ B(p50p65) heterodimer is the most abundant form, but this most likely to be due to increased expression levels of the p50 subunit or the increased affinity if the NF- κ B p50p65 heterodimer compared to other dimers. Our results suggest that I κ B α has evolved to recognize primarily p65 so that it can regulate gene expression by both p65p65 homodimers and by p50p65 heterodimers.

I κ B α binding to the NF- κ B dimer is characterized by a large negative $\Delta C_{p,obs}$

The variation of ΔH_{obs} for I κ B α /NF- κ B complex was linear over a temperature range of 17 to 33 °C (Figure 6A). At temperatures exceeding 33 °C, some negative curvature of the slope was observed (Supplementary Figure 1). A similar observation was recently been reported by Ladbury and co-workers for the phosphate 5 tetratricopeptide repeat (TPR) domain with Hsp90¹⁷. These researchers were able to show that the nonlinearity was due to an increased proportion of the unfolded form of the protein leading to an additional folding coupled binding component of the ΔH_{obs} . It is also likely that the I κ B α is unfolding in our experiments since the unfolding transition for I κ B α occurs around 45°C¹⁴. Since the TPR domain reported by Ladbury and co-workers displayed reversible thermal unfolding behavior, the data were analyzed by simultaneously fitting unfolding data and the temperature dependence of ΔH_{obs} measured by ITC. Since I κ B α displays irreversible thermal unfolding to an aggregate, we were only able to fit the linear part of the data. Determination of the $\Delta C_{p,obs}$ from the linear region of the curve has been shown to be a good approximation up to approximately 15 degrees below the unfolding temperature of the protein, as was the case in our studies¹⁷. By this method, a relatively large $\Delta C_{p,obs}$ for I κ B α binding ($-1.30 \pm 0.03 \text{ kcal mol}^{-1} \text{ K}^{-1}$) was determined.

Many attempts have been made to relate $\Delta C_{p,obs}$ to burial of polar and non polar surface area²⁵⁻²⁸ and more recently including the burial of hydroxyl groups²⁹. However, discrepancies commonly arise when folding or induced fit is coupled to binding as in the case of many protein-DNA interactions²⁸ or due the presence of large numbers of buried water molecules^{30,31}, or networks of water molecules near the surface of the interface³², or linked protonation effects^{33,34}. While we cannot rule out any of these potential mechanisms leading to such a large $\Delta C_{p,obs}$, a model of folding coupled binding of one or both binding partners could potentially account for the burial of non-polar surface area unanticipated from the structure of the complex. This is supported by our previous finding that parts of I κ B α are highly dynamic in solution¹⁴.

A coupled folding and binding mechanism is also consistent with the significant effect of removal of the fourth helix of p65 that caps ankyrin repeat 1 of I κ B α . Indeed, removal of helix four in the NLS resulted in a halving of the $\Delta C_{p,obs}$. This was a surprisingly large difference in $\Delta C_{p,obs}$ for such a small deletion from the C-terminus of NF- κ B. One likely explanation is that helix four 'caps' ankyrin repeat 1 of I κ B α effectively stabilizing the entire I κ B α molecule. If this is the case, the effect of helix four binding may be propagated by strengthening contacts between neighboring repeats of the ankyrin repeat domain of I κ B α resulting in a significant folding stabilization. This finding also has potential biological significance. With the tight association we observe for the I κ B α /NF- κ B complex, it then becomes difficult to imagine how the inhibition can be rapidly released upon IKK activation and proteasome targeting. Now one can imagine that the entire complex is brought to the proteasome, and that degradation occurs beginning with the unstructured N-terminal domain of I κ B α . Subsequent degradation of the first ankyrin repeat will then result in the loss of the interaction between helix four of p65 and I κ B α , followed by a 1000- fold increase in the k_d resulting in rapid dissociation of the complex. The rest of I κ B α could then be readily degraded and the NF- κ B could then translocate to the nucleus. Thus, helix four may represent the key to unlock the tight complex between NF- κ B and I κ B α allowing for rapid transcriptional activation upon cellular stimulation.

CONCLUSION

Direct binding experiments have revealed, for the first time, the almost irreversible binding of I κ B α to NF- κ B. This helps explain the extremely long *in vivo* half-life of the complex and the leak-proof nature of the inhibition. The thermodynamics of the NF- κ B/I κ B α interaction, together with our previous studies of the dynamics of the free I κ B α , suggest that the I κ B α /NF- κ B interaction may involve coupled folding and binding. Deletion of helix four in the NLS of

p65, which contacts the first ankyrin repeat of I κ B α , dramatically increased the dissociation rate of the complex. This observation suggests a model whereby degradation of I κ B α may only need to proceed through the first ankyrin repeat for complete and rapid NF- κ B dissociation to occur.

MATERIALS AND METHODS

Protein expression and purification.

Human I κ B α (67-287) was expressed in the Pet 11a vector and purified as previously described¹⁴. Two mutations were introduced into the p65 gene, N-terminal cysteine and a Cys38 to Ser to allow specific biotinylation only at the N-terminus. This gene will be referred to as p65. Murine NF- κ B(p50₂₄₈₋₃₇₆/p65₁₉₋₃₂₅) was co expressed in a modified pET 29b vector and purified as previously described³⁵. Cells were harvested and sonicated then centrifuged at 12,000 RPM for 45 min and the supernatant loaded onto a tandem fast flow Q and fast flow S column (GE Healthcare) equilibrated in 50 mM NaCl, 25 mM Tris, pH 7.5, 0.5 mM EDTA. After loading the Q column was disconnected and protein fractions eluted from the S with a gradient from 50 to 400 mM NaCl. Fractions were collected and analyzed by SDS PAGE. Bands were visualized by silver staining and fractions with equal intensity of P50 and p65 were collected and pooled. The final step of the purification was size exclusion on an S-200 Superdex column equilibrated in 150 mM NaCl, 10 mM MOPS, pH 7.5, 0.5 mM EDTA, 0.5 mM sodium azide. The purified fractions were biotinylated by incubation with a 1:1 molar ratio of biotin PEO maleimide (Pierce Chemicals), at room temperature for 30 min and purified immediately by size exclusion chromatography on an S200 Superdex 16/60 column. Fractions containing the biotinylated heterodimer were collected and stored at -80 °C in 50 μ l portions until used.

All other NF- κ B constructs were expressed using a Pet 11a single expression vector and purified using a similar tandem column technique. Again, an N-terminal cysteine was introduced into the p65 as described above. For the NF- κ B p65₁₉₀₋₃₂₁ and NF- κ B p50₂₄₈₋₃₅₀ constructs, *E. coli* BL21 DE3 cells were grown to an OD of 0.6 and induced at room temperature for 16 hours with 0.1mM IPTG. For the NF- κ B p65₁₉₀₋₃₂₁ the column was equilibrated in 50mM NaCl, 25 mM MES pH 7.0, 10 mM BME, 0.5 mM EDTA. For the NF- κ B p50₂₄₈₋₃₅₀ the column was equilibrated in 50 mM NaCl, 25 mM MES pH 6.2, 10 mM BME, 0.5 mM EDTA and gradient of 50-300 mM NaCl, was run.

For the NF- κ B p65₁₋₃₂₅, NF- κ B p65₁₋₃₀₄ and NF- κ B p50₃₉₋₃₆₃ proteins, cells were induced with 0.5 mM IPTG. NF- κ B p65₁₋₃₂₅ the columns were equilibrated in 50 mM NaCl, 25 mM MES, pH 6.5, 10 mM BME, 0.5 mM EDTA, a gradient was run from 50-450 mM NaCl. For NF- κ B p65₁₋₃₀₄ 50 mM NaCl, 25 mM Tris, pH 7.0, 10 mM BME, 0.5 mM EDTA and a 50-300 mM gradient was used. For NF- κ B p50₃₉₋₃₆₃ the columns were equilibrated in 50 mM NaCl, 25 mM MES, pH 6.2, 10 mM BME, 0.5 mM EDTA. Gradient was run from 50-700 mM NaCl.

Protein concentrations were determined spectrophotometrically from a scan of wavelengths 340-220 nm using the following ϵ_{280} values: 24180 for NF- κ B p50₂₄₈₋₃₅₀, 21620 for NF- κ B p65₁₉₀₋₃₂₁, 19060 for NF- κ B p65₁₉₀₋₃₀₄, 36980 for the NF- κ B p65₁₋₃₂₅, 34420 for NF- κ B p65₁₋₃₂₅, and 42100 for NF- κ B p50₃₉₋₃₆₃ homodimers and 30580 for NF- κ B(p50₂₄₈₋₃₇₆/p65₁₉₋₃₂₅), 22900 for NF- κ B(p50₂₄₈₋₃₅₀/p65₁₉₀₋₃₂₁), 21620 for NF- κ B(p50₂₄₈₋₃₅₀/p65₁₉₀₋₃₀₄), 39540 for NF- κ B(p50₃₉₋₃₆₃/p65₁₋₃₂₅), 38260 for NF- κ B(p50₃₉₋₃₆₃/p65₁₋₃₀₄) heterodimers. For I κ B α ₆₇₋₂₈₇ an ϵ_{280} of 12090 was used. Dimers were formed *in vitro* by incubating an equimolar amount for two hours at 25 °C and overnight at 4 °C prior to ITC experiments. For SPR experiments, the p65 was biotinylated as already described. A 1000 fold excess of un-biotinylated p50 (for heterodimers) or p65 (for homodimers) was incubated with biotinylated p65 and the equilibrated mixture was immobilized immediately on a streptavidin (SA) SPR chip.

The C-terminal residues 289-320 of NF- κ B were expressed in the trp leader vector which contains an octa-histidine tag and thrombin cleavage sequence and drives small peptides into inclusion bodies³⁶. Inclusion bodies were solubilized 6M guanidine hydrochloride, 50mM Tris, pH 7.4 and the solubilized peptide was captured by Ni-NTA column equilibrated in the same buffer, and a gradient was run to a final concentration of 150 mM NaCl, 50 mM Tris, pH 7.4, 2mM CaCl₂. The peptide was cleaved from the column with thrombin on the column for 4 hrs at 25 °C. The final purification step was reverse phase HPLC on a C18 column with a 0-50% acetonitrile gradient, with 0.1% TFA. The peptide was lyophilized and dissolved in 150mM NaCl, 10mM MOPS, pH 7.5, 0.5mM EDTA and the pH adjusted with 10 M NaOH.

SPR experiments

Sensorgrams were recorded on a Biacore 3000 instrument using streptavidin (SA) chips. Biotinylated NF- κ B was immobilized on the chip in a high salt buffer (500 mM NaCl, 10mM Tris, pH 7.5, 0.5mM EDTA, 0.5mM sodium azide, 0.005% P20). Sensorgrams were run in the automatic subtraction mode using flow cell 1 (FC 1) as an unmodified reference. Data was collected for FC's 2,3 and 4, which contained varying amounts of NF- κ B ligand with the lowest amount immobilized on FC2 and the highest on FC4. Injections were made using the kinject injection mode, alternating highest with lowest concentration samples, with a 5 minute contact time and a 1200 second dissociation phase, in all cases except for the weaker interactions where a 3 min contact time and a 3 min dissociation phase was used. The running buffer used for the binding experiments was 150 mM NaCl, 10mM Tris, pH 7.5, 10% (w/v) glycerol, 3mM DTT, 0.5mM sodium azide, 0.2 mM EDTA and 0.005% P20. The glycerol improved the stability of the NF- κ B during regeneration. Regeneration was achieved using a one min pulse of a urea solution. The concentration of urea required depended on the NF- κ B construct and the experimental temperature and was prepared by diluting a 6M stock into the running buffer. The minimum urea concentration required for complete regeneration under each condition was determined by repeat injections. The data was analyzed using the Bia Evaluation 4.1 software using a simple 1:1 langmuir binding model. Between 3 and 12 sensorgrams were obtained for each construct and condition tested using a range of immobilized NF- κ B and I κ B α concentrations.

For NF- κ B(p50₂₄₈₋₃₇₆/p65₁₉₋₃₂₅ at 37 °C, 200, 300 and 400 RU of NF- κ B were immobilized. 0.23 to 4.0 nM I κ B α was injected. A one min pulse of 3M urea was used for regeneration. Lower ligand and analyte concentrations were not employed to avoid the long term noise of the instrument becoming significant relative to the slow dissociation of the I κ B α ³⁷.

For NF- κ B(p50₂₄₈₋₃₅₀/p65₁₉₀₋₃₂₁ sensorgrams were obtained at 25 and 37 °C. NF- κ B was immobilized at 50, 75, 100, 150, 200, 250 and 350 RUs. Sensorgrams were recorded using several ranges of I κ B α . These were: 0.87 to 9.9 nM I κ B α with 200, 250 and 350 RU NF- κ B, 0.24 to 20 nM I κ B α for 50, 75 and 100 RU of NF- κ B and with 0.01 to 5 nM I κ B α with 100, 150 and 200 RU of NF- κ B at 37 °C. For experiments at 25 °C, concentrations used were 0.87 to 9.9 nM I κ B α with 100, 200 and 300 RU NF- κ B, 0.24 to 20 and 0.022 to 10 nM I κ B α with 50, 75 and 100 RU NF- κ B. A one min pulse of 1.5 M urea was used for regeneration at 37 °C and 3 M Urea at 25 °C.

For NF- κ B(p50₂₄₈₋₃₅₀/p65₁₉₋₃₀₄ at 25 °C, 16 to 1000 nM I κ B α was used with 100, 200 and 250 RU of NF- κ B, and 9.9 to 5000 nM I κ B α was used with 50, 75 and 100 RU of NF- κ B. At 15 °C, 9.9 to 5000 nM I κ B α was used with 50, 75 and 100 RU of NF- κ B. No regeneration was required at either temperature with this NF- κ B construct because it bound so weakly. Data were analyzed by equilibrium analysis in addition to the kinetic analysis. The equilibrium response was plotted against the I κ B α concentration and a line was fit to $R = K_A \times [I\kappa B\alpha] \cdot R_{\max} / (K_A \cdot [I\kappa B\alpha] + 1)$ where R is the equilibrium response at a specific I κ B α concentration, $R_{\max}$ is the response at saturation of the ligand on the chip and $K_A = 1/K_D$.

For the NF- κ B(p50₂₄₈₋₃₅₀/p65₁₉₋₃₀₄), the effect of the 10% glycerol in the running buffer was assessed by experiments at 25 °C with 9.9 to 5000 nM I κ B α and 50, 75 and 100 RU of NF- κ B immobilized using a running buffer that was the same as that used for the ITC experiments (150 mM NaCl, 10 mM MOPS, pH 7.5, 0.5 mM EDTA, 0.5 mM sodium azide, 0.005% p20). No significant difference in the binding data was observed using this buffer.

ITC experiments

ITC experiments were carried out on a Microcal MCS instrument. I κ B α and NF- κ B were purified by size exclusion chromatography on an S-75 or S-200 column respectively immediately prior to use. In a typical ITC experiment, 20 15 μ l injections of 50 μ M NF- κ B were made into a 5 μ M I κ B α solution in the cell. ITC experiment were carried out in a buffer of 150 mM NaCl, 10 mM MOPS, pH 7.5, 0.5 mM EDTA, 0.5 mM sodium azide. Isotherms were analyzed using the Origin software (Microcal) as described elsewhere³⁸. For the very tight complexes, the $K_{D,obs}$ could not be determined due the high 'c' value for the interaction, where c is defined by Wiseman *et al.*³⁸.

Surface area calculations

Surface area calculations were carried out using the Getarea 1.1 program at www.scsb.utmb.edu/cgi-bin/get_a_form.tcl³⁹.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A) Ribbon diagram showing the x-ray crystal structure of the NF- κ B/I κ B α complex. I κ B α is colored blue. The NF- κ B is colored to show the p50 subunit in green, the p65 RHR and dimerization domain in red and the p65 NLS and NLS extension in violet. This figure was created using PyMOL v. 0.97 (<http://pymol.sourceforge.net/>) for the PDB coordinates (PDB accession code: 1nfi)⁴⁰. B) Schematic to show the domain structure of the NF- κ B p50 (green) p65 (red) heterodimer.

Figure 2.

Summary of SPR data collected for the NF-κB/IκBα interaction. In all experiments the NF-κB was immobilized to a streptavidin chip by a biotin tag on the p65 N terminus and IκBα was flowed over at a flow rate of 50 μl per minute. A) Sensorgrams of 0.23 to 4.0 nM IκBα flowed over NF-κB(p50₂₄₈₋₃₇₆/p65₁₉₋₃₂₅) at 37 °C. B) Sensorgrams of 0.87 to 6.6 nM of IκBα flowed over NF-κB(p50₂₄₈₋₃₅₀/p65₁₉₀₋₃₂₁) at 37 °C. C) Sensorgrams of 0.244 to 250 nM of IκBα flowed over NF-κB(p50₂₄₈₋₃₅₀/p65₁₉₀₋₃₂₁) at 25 °C. D) Binding isotherm of 50 μM NF-κB (p50₂₄₈₋₃₇₆/p65₁₉₀₋₃₂₁) titrated in 5 μl injections into 3 μM IκBα. The buffer was 150 mM NaCl, 10 mM MOPS, pH 7.5, 0.5 mM EDTA, 0.5 mM sodium azide and the temperature was 31 °C. Data were analyzed using a model for a single set of identical binding sites after the heats

of dilution of NF- κ B into buffer were subtracted. $K_{D,obs}$ was $2.2 \text{ nM} \pm 0.6 \text{ nM}$, and the stoichiometry was 0.9. The large error in K_D was due to the high 'c' value for the interaction, where c is defined by Wiseman *et al.* and it is likely that affinity is significantly underestimated³⁸.

Figure 3.

Summary of SPR data for the NF-κB(p50₂₄₈₋₃₅₀/p65₁₉₀₋₃₀₄) from which the p65 helix four (305-321) was deleted. A) Sensorgrams of 9.8 to 5000 nM concentrations of IκBα flowed over NF-κB at 25 °C kinetic analysis using a simple 1:1 model yielded a $K_{D,obs}$ of 460 ± 120 nM, B) equilibrium analysis yielded a $K_{D,obs} = 410 \pm 100$ nM C) sensorgrams of 9.8 to 5000 nM concentrations of IκBα flowed over NF-κB 15 °C. Kinetic analysis yielded a $K_{D,obs} = 158 \pm 12$, D) equilibrium analysis yielded a $K_{D,obs} = 210 \pm 93$ nM. The equilibrium response was plotted against the IκBα concentration and a line was fit to $R = K_A \times [I\kappa B\alpha] \cdot R_{\max} / (K_A \cdot [I\kappa B\alpha] + 1)$ where R is the equilibrium response at a specific IκBα concentration, $R_{\max}$ is the response at saturation of the ligand on the chip and $K_A = 1/K_D$.

Figure 4.

ITC binding isotherms for NF- κ B binding to I κ B α in 150 mM NaCl, 10 mM MOPS, pH 7.5, 0.5 mM EDTA, 0.5 mM sodium azide at 30°C. Experiments were carried out in triplicate and data were analyzed using a model for a single set of identical binding sites after the heats of dilution of NF- κ B into buffer were subtracted. A) NF- κ B(p50₂₄₈₋₃₅₀/p65₁₉₀₋₃₀₄), $K_{D,obs} = 333 \pm 21 \times 10^{-9}$ M; $n = 1.1 \pm 0.01$ B) NF- κ B(p50₂₄₈₋₃₅₀/p65₁₋₃₀₄) $K_{D,obs} = 125 \pm 8 \times 10^{-9}$ M; $n = 0.99 \pm 0.02$ and C) NF- κ B(p50₃₉₋₃₆₃/p65₁₋₃₀₄) $K_{D,obs} = 42 \pm 6 \times 10^{-9}$ M; $n = 0.84 \pm 0.01$.

Figure 5.

ITC binding isotherm for the peptide fragment of NF- κ B p65(289-320) binding to I κ B α , in 150 mM NaCl, 10 mM MOPS, pH 7.5, 0.5 mM EDTA, 0.5 mM sodium azide at 30°C. Data were analyzed using a model for a single set of identical binding sites after the heats of dilution of NF- κ B into buffer was subtracted a $K_{D,obs}$ of $1.3 \pm 0.09 \times 10^{-6}$ M and a stoichiometry of 1.1 ± 0.05 at 30 °C was determined.

Figure 6.

A) Extrapolation of the thermodynamic characteristics of the NF- κ B(p50₂₄₈₋₃₅₀/p65₁₉₀₋₃₀₄)/I κ B α interaction using the Gibbs-Helmholtz relation in the form: $\Delta G_{\text{bind}}^{\circ}(T_o) = \Delta H(T_o) - T_o[\Delta H(T) - \Delta G^{\circ}(T)]/T + \Delta C_p \ln(T_o/T)$, where ΔH is the enthalpy change, $\Delta G_{\text{bind}}^{\circ}$ is the free energy change upon binding, $T_o = 298$ K, and T is the absolute temperature. The ΔG_{obs} of the interaction is most favorable at approximately 20 °C when $T\Delta S_{\text{obs}} = 0$ (T_S). Since T_S occurs the midpoint of the experimental range of temperatures investigated the variation in $K_{D,\text{obs}}$ appears small compared to experimental error. B) Temperature dependence of the ΔH_{obs} for the I κ B α / NF- κ B (p50₂₄₈₋₃₅₀/p65₁₉₀₋₃₂₁) (blue) and NF- κ B-NLS truncation (p50₂₄₈₋₃₅₀/p65₁₉₀₋₃₀₄) (red) interaction. The slope of the line was used to determine a $\Delta C_{p,\text{obs}}$ of -1.30 ± 0.03 for the full length NLS and -0.60 ± 0.03 for the NLS truncated NF- κ B complex with I κ B α .

Figure 7.

Temperature dependence of the ΔH_{obs} for the $\text{IkB}\alpha/\text{NF-}\kappa\text{B}$ ($\text{p50}_{248-350}/\text{p65}_{190-321}$) (blue) and NF- κB p65 homodimer ($\text{p65}_{190-321}/\text{p65}_{190-321}$) (green) interaction. The trend shows that binding of the p65 homodimer to $\text{IkB}\alpha$ has a consistently more favorable ΔH_{obs} relative to the heterodimer over the experimental temperature range.

Table 1
SPR kinetic and thermodynamic values for interactions between NF-κB and IκB constructs.

A. Interaction of NF-κB(p50 ₂₄₈₋₃₇₆ /p65 ₁₉₀₋₃₂₅) with IκBα(67-287) and IκBα(67-317)					
Interaction	k_a ($\times 10^6 \text{ M}^{-1} \text{ s}^{-1}$)	k_d ($\times 10^{-4} \text{ s}^{-1}$)*	$K_{A,obs}$ ($\times 10^{10} \text{ M}^{-1}$)	$K_{D,obs}$ ($\times 10^{-11} \text{ M}$)	χ^2
67-287	3.7 ± 0.16	1.5 ± 0.16	2.5 ± 0.36	3.9 ± 0.30	1.0
67-317	3.3 ± 0.60	1.4 ± 0.55	2.2 ± 0.10	4.5 ± 1.0	0.7
B. Interaction of NF-κB(p50 ₂₄₈₋₃₅₀ /p65 ₁₉₀₋₃₂₁) with IκBα(67-287) as a function of temperature					
Temperature	k_a ($\times 10^6 \text{ M}^{-1} \text{ s}^{-1}$)	k_d ($\times 10^{-3} \text{ s}^{-1}$)*	$K_{A,obs}$ ($\times 10^9 \text{ M}^{-1}$)	$K_{D,obs}$ ($\times 10^{-9} \text{ M}$)	χ^2
37	1.7 ± 0.32	0.54 ± 0.099	3.3 ± 0.93	0.32 ± 0.10	5.5
25	1.3 ± 0.18	0.12 ± 0.041	0.12 ± 0.034	0.091 ± 0.027	1.4
C. Interaction of NF-κB(p50 ₂₄₈₋₃₅₀ /p65 ₁₉₀₋₃₀₄) with IκBα(67-287)					
Temperature	k_a ($\times 10^6 \text{ M}^{-1} \text{ s}^{-1}$)	k_d ($\times 10^{-3} \text{ s}^{-1}$)	$K_{A,obs}$ ($\times 10^9 \text{ M}^{-1}$)	$K_{D,obs}$ ($\times 10^{-9} \text{ M}$)	χ^2
25	0.24 ± 0.08	100 ± 20	0.0023 ± 0.00056	460 ± 120	0.43
15	0.15 ± 0.01	24 ± 0.95	0.0064 ± 0.0005	158 ± 12	0.28
D. Interaction of NF-κB(p50/p65) heterodimer and NF-κB(p65/p65) homodimer binding to IκBα					
Interaction	k_a ($\times 10^6 \text{ M}^{-1} \text{ s}^{-1}$)	k_d ($\times 10^{-3} \text{ s}^{-1}$)*	$K_{A,obs}$ ($\times 10^9 \text{ M}^{-1}$)	$K_{D,obs}$ ($\times 10^{-9} \text{ M}$)	χ^2
p50p65 ^a	1.7 ± 0.32	0.54 ± 0.10	3.3 ± 0.93	0.32 ± 0.10	5.5
p65p65 ^b	2.9 ± 0.30	1.6 ± 0.14	1.9 ± 0.25	0.54 ± 0.08	0.45

¹Data were fit using the Biaevaluation 4.1 software to a simple Langmuir binding model. Experiments were carried out in triplicate for estimations of errors. In each experiment the amount of immobilized NF-κB was varied in the range of 50 to 400 RU.

^aConstruct used was NF-κB(p50₂₄₈₋₃₅₀/p65₁₉₀₋₃₂₁) and p65₁₉₀₋₃₂₁/p65₁₉₀₋₃₂₁.

^bConstruct used was NF-κB(p65₁₉₀₋₃₂₁/p65₁₉₀₋₃₂₁).

* Since the dissociation rate was very low, we did not cover even a single half life during the 20 min dissociation period. This means that the dissociation rate we determine represents the upper limit of the true k_d and the $K_{D,obs}$ for the complex may be even lower than we are able to determine.

Table 2
Summary of SPR and ITC experiments probing C-terminal truncation of p65.

Schematic	Temp. (°C)	$K_{D,obs-SPR}(\times 10^{-9}M)$
37	0.32 ± 0.10
25	0.091 ± 0.027

Schematic	Temp. (°C)	$K_{D,obs-SPR}(\times 10^{-9}M)$
25	460 ± 120
30	ND
30	ND	

Schematic	Temp. (°C)	$K_{D,obs-SPR}(\times 10^{-9}M)$
30	ND
17	ND	

Schematic	Temp. (°C)	$K_{D,obs-SPR}(\times 10^{-9}M)$
15	158 ± 12

SPR data were fit using the Biaevaluation 4.1 software to a simple Langmuir binding model. Experiments were carried out in triplicate for estimations of errors. In each experiment the amount of immobilized NF-κB was varied in the range of 50 to 400 RU. ITC data were fit to a model for a single set of identical binding sites after the heats of dilution of NF-κB into buffer was subtracted. The stoichiometry for the interaction was found to be consistent with a one-to-one binding model in all cases. For the SPR experiments the experimental temperature depended on the dissociation rate for the interaction. For the very tight binding complexes at the top of the table the dissociation rate was too low to be quantified at temperatures below 37 °C. For ITC experiments, the data could not be obtained easily at temperature below 20 °C due to the small ΔH_{obs} and limitations of the instrument.

Table 3

Comparison of NF-κB(p50/p65) heterodimer and NF-κB(p65/p65) homodimer binding data from SPR and ITC experiments.

	Temperature °C	$K_{D-SPR} (\times 10^{-9}M)$
37	0.32 ± 0.10

Schematic	Temperature °C	K_{D-SPR} ($\times 10^{-9}M$)
37	0.54 ± 0.08
30	ND

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Schematic	Temperature °C	K_{D-SPR} ($\times 10^{-9}M$)
30	ND
30	ND
30	ND
30	ND

SPR data were fit using the Biaevaluation 4.1 software to a simple Langmuir binding model. Experiments were carried out in triplicate for estimations of errors. In each experiment the amount of immobilized NF- κ B was varied in the range of 50 to 400 RU. ITC data were fit to a model for a single set of identical binding sites after the heats of dilution of NF- κ B into buffer was subtracted. The stoichiometry for the interaction was found to be consistent with a one-to-one binding model in all cases. For the SPR experiments the experimental temperature depended on the dissociation rate for the interaction. For the very tight binding complexes at the top of the table the dissociation rate was too low to be quantified at temperatures below 37 °C. For ITC experiments the data could not be obtained easily at temperature below 20 °C due to the small ΔH_{obs} and limitations of the instrument.

Table 4

Summary of the free energy change (ΔG_{obs}) for different construct of NF- κ B p50p65 with I κ B α .

Schematic	Temp. (°C)	Δ SASA (Å) ^a
37	-4902

Schematic	Temp. (°C)	Δ SASA (Å) ^a
37	-4036

ct

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Schematic	Temp. (°C)	Δ SASA (Å) ^a
25	-4036

ct

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Schematic	Temp. (°C)	$\Delta SASA$ (Å) ^a
25	-3218
25	-1871

Schematic	Temp. (°C)	Δ SASA (Å) ^a
30	-3218
30	-1871

^aThe Δ SASA (Å) was determined using the program GetArea 1.1 (http://www.scsb.utmb.edu/cgi-bin/get_a_form.tcl)³⁹.