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Exclusivity and Compensation in NF κ B Dimer distributions and I κ B inhibition

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Abstract

The NF κ B transcription factor family members RelA, p50, and cRel form homo and heterodimers that are inhibited by I κ B α , I κ B β , and I κ B ϵ . These NF κ B family members have diverse biological functions and their expression profiles differ leading to different concentrations in different tissue types. Here we present definitive biophysical measurements of the NF κ B dimer affinities and inhibitor affinities to better understand dimer exchange and how the presence of inhibitors may alter the equilibrium concentrations of NF κ B dimers in the cellular context. Fluorescence anisotropy binding experiments were performed at low concentrations to mimic intracellular concentrations. We report much stronger binding affinities than had been previously reported by non-equilibrium gel-shift and analytical ultracentrifugation assays. The results reveal a wide range of NF κ B dimer affinities and a strong preference of each I κ B for a small subset of NF κ B dimers. Once the preferred I κ B is bound, dimer exchange no longer occurs over a period of days. A mathematical model of the cellular distribution of these canonical NF κ B transcription factors based on the revised binding affinities recapitulates intracellular observations and provides simple, precise explanations for observed cellular phenomena.

Keywords

Protein binding affinity; NF κ B signaling system; ankyrin repeat protein; fluorescence anisotropy; inhibitors of NF κ B signaling

Introduction

The nuclear factor kappa B (NF κ B) family of transcription factors responds to a large number of extracellular stress stimuli, including factors controlling inflammation and the

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Supporting Information

Two notes detailing the Matlab equations and fitting

immune response (1–3). Dysregulation of NF κ B results in numerous disease states, particularly cancer (4). NF κ B is a member of the Rel homology domain-containing (RHD) family of proteins which are composed of two immunoglobulin-like (Ig-like) β -barrel subdomains connected by a linker. The family of NF κ B proteins includes RelA, p50, cRel, RelB, and p52, which form homo- and heterodimers. Utilizing mostly irregular loops, both Ig-like subdomains of both monomers engage the DNA major groove. The N-terminal Ig-like domain is typically referred to as the DNA-binding domain due to its large surface engaged in DNA-binding. The C-terminal Ig-like domain is responsible for dimerization and is therefore referred to as the dimerization domain. RelA, cRel and RelB contain transcriptional activation domains whereas p50 and p52 do not. Therefore, neither p50 nor p52 homodimers are not capable of transcription activation but heterodimers of p50 can activate transcription. The population of various NF κ B dimers changes dramatically during cell differentiation. RelA-p50 is most abundant in the early lineages of B-cell lines, whereas p50-cRel dimers are present in later lineages (5). In murine embryonic fibroblasts, RelA-p50 and RelA homodimer are observed and appear to activate different sets of genes (6). The p50-cRel heterodimers, rather than RelA, play a key role in B-cell proliferation (7, 8). In B-cells, I κ B ϵ plays a specific role in limiting cRel and RelA-containing dimers (9).

The NF κ B family of transcription factors is highly regulated because NF κ B dimers respond to extracellular stress responses by turning on hundreds of genes. Studies of the most abundant, canonical RelA-p50 dimer reveal that all dimers are kept in the cytoplasm by a bound inhibitor that requires signal-mediated degradation for removal (3, 10). Once a stress signal is received, the response is strong because the concentration of free I κ B α is very low due to the rapid degradation rate of free I κ B α (2, 11). Once I κ B α is degraded, the nuclear localization signal on the NF κ B is unmasked and promotes nuclear translocation.

Our lab discovered that an equally stringent system is in place for signal termination. In a classical negative feedback loop, nuclear NF κ B activates transcription of new I κ B α , which translocates to the nucleus and mediates NF κ B removal from the DNA in a rapid and complete kinetic process (12, 13). This process, which we have termed “molecular stripping”, makes molecular sense because free I κ B α would have to compete for NF κ B binding with thousands of DNA target sites. In cells, the rate of molecular stripping has been shown to directly control the rate of export of NF κ B from the nucleus returning the system to the “off” state (14). Without this kinetically controlled stripping process, the termination of NF κ B transcription would be incomplete and leaky whereas with stripping every gene transcription event is terminated and all of the NF κ B is removed from the nucleus rapidly and completely (15, 16).

But what about other dimers and inhibitors? RelA homodimer appears to be the most unstable dimer, but is stabilized by I κ B β (17). In fact, in I κ B β ^{-/-} cells, RelA homodimer is no longer observed. Thus, in addition to I κ B α being able to remove NF κ B from the nucleus, it appears that I κ B β may stabilize the RelA homodimer (17).

A complete understanding of such a complex system which is under kinetic control, requires rigorous measurement of binding affinities of all protein complexes. While some attempts have been made at such measurements, there is much disagreement in the field. Indeed, gel

shift assays first estimated the affinity between NF κ B and I κ B α to be 1 nM (18). However, later isothermal titration calorimetry and surface plasmon resonance estimated the affinity at 30 pM, and this much tighter binding was more consistent with the long intracellular half-life of the complex, which was estimated at 48 hrs in IKK^{-/-} cells (11). Previous reports estimated the RelA homodimer binding affinity at 800 nM (17). Such a weak binding affinity would suggest a rapid dimer equilibrium resulting in a substantial monomer concentration at the cytoplasmic concentration of 350 nM. The observation of such a weak binding led to the suggestion that I κ B β may bind to monomeric RelA and perhaps facilitate dimerization (17).

We set out to develop a more rigorous binding experiment that could measure the equilibria for dimer exchange and I κ B binding at concentrations in the nM range, which is more in line with intracellular concentrations. We found that the binding affinities were much tighter than previously reported, and we present a modified mathematical model incorporating the new data.

Materials and Methods

Protein expression and purification.

Murine, N-terminal hexahistidine-p50_{39–350}/RelA_{19–321} heterodimer (full-length NF κ B) was co-expressed as described previously (19). This co-expression system was also used for the cRel-p50 and cRel-RelA heterodimers. Essentially, one subunit is over-expressed and the other has an N-terminal His₆-tag so that only heterodimer is purified using nickel affinity chromatography. The heterodimers were further purified by cation exchange chromatography (MonoS 10/10; GE healthcare) using a 0–700 mM NaCl gradient in 25mM Tris pH 7.5, 0.5 mM EDTA, 1mM DTT and finally size exclusion chromatography (Superdex S200; GE healthcare) in 25mM Tris pH 7.5, 150mM NaCl, 0.5mM EDTA, 1mM DTT. Most of the experiments were performed with fresh proteins preparations. Murine p50_{39–350}/cRel_{1–294} and cRel_{1–294}/RelA_{19–321} were purified as with p50-RelA.

NF κ B homodimers (RelA_{19–321}/RelA_{19–321}, p50_{39–350}/p50_{39–350}, and cRel_{1–294}/cRel_{1–294}) expressed similarly to the NF κ B heterodimers. The NF κ B homodimers were purified by cation exchange chromatography (SP Sepharose Fast Flow; Sigma Aldrich) via a linear gradient of 0–700 mM NaCl in 25 mM Tris pH 7.5, 0.5 mM EDTA, 0.5 mM PMSF, and 10 mM β ME. Fractions containing NF κ B homodimers were pooled and dialyzed overnight at 4 °C against buffer containing 25 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT and frozen in aliquots at –80 °C. Immediately before use, the proteins were purified by MonoS cation exchange and S200 gel filtration chromatography as described for the NF κ B heterodimers.

I κ B proteins were expressed and purified as previously described (20) using nickel affinity chromatography for I κ B ϵ and anion exchange chromatography for I κ B α and I κ B β , and a final gel filtration chromatography purification step for all (Superdex S75 for I κ B α and Superdex S200 for I κ B ϵ and I κ B β ; GE Healthcare) (20).

Protein concentrations were determined by measuring the absorbance at 280 nm using the following molar extinction coefficients (ExPASy): RelA_{19–321}/RelA_{19–321}: 36980 M⁻¹cm⁻¹, p50_{39–350}/RelA_{19–321}: 39540 M⁻¹cm⁻¹, cRel_{1–294}/RelA_{19–321}: 46395 M⁻¹cm⁻¹, p50_{39–350}/p50_{39–350}: 42100 M⁻¹cm⁻¹, p50_{39–350}/cRel_{1–294}: 49125 M⁻¹cm⁻¹, cRel_{1–294}/cRel_{1–294}: 50990 M⁻¹cm⁻¹, RelA_{191–321}/RelA_{191–321}: 21620 M⁻¹cm⁻¹, p50_{245–363}/p50_{245–350}: 24180 M⁻¹cm⁻¹, IκBα_{67–287}: 12090 M⁻¹cm⁻¹, IκBβ_{50–359}: 15930 M⁻¹cm⁻¹, (His)₆-IκBε_{40–364}: 16960 M⁻¹cm⁻¹.

Preparation of fluorescently labeled NFκB subunits.

For the steady-state anisotropy experiments to determine NFκB dimerization affinities, an N-terminal cysteine (NCys) residue was introduced on RelA_{190–321} or p50_{248–350} and for the steady-state anisotropy experiments to determine IκB binding affinities, an N-terminal cysteine (NCys) residue was introduced on RelA_{19–321} or p50_{39–350} using site directed mutagenesis as previously described (21). These NCys constructs were purified exactly as described for wild-type NFκB dimers. Each NCys NFκB dimer was purified using gel filtration (Superdex S75 for RelA_{190–321} and p50_{248–350} and Superdex S200 for RelA_{19–321} and p50_{39–350}; GE Healthcare) in 25 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM EDTA. The purified N-Cys-NFκB (10–20 μM in 500 μL) was then incubated with 500 of immobilized Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) resin (Thermo-Fisher) for 10 minutes at 4 °C with rocking to reduce all cysteine residues. Resin was pelleted via centrifugation at 1000 ×g for 1 minute and the supernatant was transferred to a fresh tube. A 25-fold molar excess of thiol- reactive maleimide-Oregon Green 488 (Sigma-Aldrich) was added to the reduced NFκB protein and incubated with rocking at 4 °C overnight. The next morning the labeled protein was purified from un-conjugated dye using a NAP-10 column pre-packed with Sephadex G-25 resin (GE Healthcare) previously equilibrated in Dialysis Buffer. The concentration of NFκB was determined by correcting the absorbance at 280 nm (A₂₈₀) for the absorbance of the fluorophore (A₄₉₅) using the following equation:

Where 0.12 is a correction factor to account for the absorbance of Oregon Green at 280 nm and $\epsilon_{\text{NF}\kappa\text{B}}$ is the molar extinction coefficient for the NFκB dimer being used.

The degree of labeling was determined from the absorbance at 495 nm (A₄₉₅) using the following equation:

$$\text{Degree of Labeling} = \frac{A_{495}}{70000 \times [\text{NF}\kappa\text{B}]}$$

Where is the, 70,000 is the molar extinction coefficient of Oregon Green 488 at 495 nm in units of M⁻¹cm⁻¹, and [NFκB] is the concentration of the NFκB dimer in M. The proteins used in the anisotropy experiments had 0.8 – 1 mol of label per mol of protein, and they were used immediately after preparation and final size exclusion purification. We hereafter denote the Oregon Green 488 labeled proteins as follows: RelA_{190–321}: *RelA_{dd}, p50_{245–350}: *p50_{dd}, RelA_{19–321}: *RelA_{FL}, and p50_{39–350}: *p50_{FL}.

Steady-state fluorescence anisotropy experiments.—To measure NFκB dimer binding affinities, we devised a method in which the concentration of either *RelA_{dd} or

*p50_{dd} was held constant at 100 pM and different concentrations of an unlabeled full-length NFκB dimer (1 pM – 100 nM depending on the affinity determined from preliminary assays) were added. For the

$$[\text{NF}\kappa\text{B}] = \frac{A_{280} - (A_{495} \times 0.12)}{\epsilon_{\text{NF}\kappa\text{B}}}$$

anisotropy experiments to determine the affinities of IκBs for NFκB dimers, the concentration of the *NFκB was held constant at 10pM (RelA-RelA), 500 pM (RelA-p50), 5 nM (RelA-cRel), 2.5 nM for p50-p50, 5nM for p50-cRel, and 1nM for cRel-cRel. Concentrations of IκBα_{67–287}, IκBβ_{39–363}, and IκBε_{40–364} were varied from 5 pM to 50 nM. Experiments were repeated three to five times on different days with fresh protein each time.

Anisotropy values were determined by reading the fluorescence polarization on a Beckman Coulter DTX 880 Multimode Detector plate reader with polarized excitation at 485 nm and two emission filters at 535 nm equipped with parallel and perpendicular polarizers. Readings were performed with a 1 s integration time utilizing a G-factor of 0.67. After obtaining the parallel and perpendicular fluorescence readings, anisotropy values were determined using the following equation:

$$R = \frac{I_{V,V} - (G \times I_{V,H})}{I_{V,V} + (2 \times G \times I_{V,H})}$$

Where R is the anisotropy value in units of milli-polarization (mP), $I_{V,V}$ is the fluorescence intensity in the parallel direction, $I_{V,H}$ is the fluorescence intensity in the perpendicular direction, and G is the instrumental G-factor of 0.67. Anisotropy values were plotted as a function of increasing concentration of the unlabeled binding partner after subtracting the initial anisotropy at 0 ligand concentration. Data were fit to the following equation using Kaleidagraph 4.5 to solve for the K_D .

$$\text{Anisotropy} = \frac{\text{Anisotropy}_{\text{max}}[x]}{K_D + [x]}$$

where x is the concentration of unlabeled dimer.

Due to the fact that the dimer affinities were of the same order of magnitude as the concentrations of labeled species, a numerical strategy implemented in MATLAB (version R2016a) was used to fit the fluorescence anisotropy data for NFκB dimers. We began by measuring the dimer affinity of the RelA homodimer, which was thought to be the weakest dimer. Then, using the K_D we determined for the RelA homodimer the remaining K_D s from the heterodimer experiments were determined by first generating an initial K_D from fitting the data from each experiment to the standard equilibrium equation. Based on this initial value a wide range of possible values for the K_D was generated. For each guess, equilibrium concentrations of all species were numerically calculated to simulate a binding curve

(fraction bound vs. full-length dimer concentration). The simulated binding curve was then compared to the fluorescence anisotropy data to determine the K_D that best fit as determined by the correlation coefficients. Finally, the simulated binding curve was converted to a simulated anisotropy change by multiplying with a coefficient determined by least squares between experiment and calculation.

In the homodimer anisotropy experiments, three reactions and five species were involved: $*N_{dd} - *N_{dd} \rightleftharpoons 2 *N_{dd}$, $N - N \rightleftharpoons 2N$, and $*N_{dd} - N \rightleftharpoons *N_{dd} + N$ where $*N_{dd}$ where $*N_{dd}$ and N denote for the labeled dimerization domain and the full-length subunit of RelA or p50. All three reactions were assumed to have the same K_D (to be determined). In the RelA-p50 heterodimer experiment, six reactions and nine species were involved:

$*RelA_{dd} - *RelA_{dd} \rightleftharpoons 2 *RelA_{dd}$, $*RelA_{dd} - RelA \rightleftharpoons *RelA_{dd} + RelA$, $RelA - RelA \rightleftharpoons 2 RelA$, $*RelA_{dd} - p50 \rightleftharpoons *RelA_{dd} + p50$, $RelA - p50 \rightleftharpoons RelA + p50$,

$p50 - p50 \rightleftharpoons 2 p50$. In this case, the K_D s for the homodimers from the previous fitting of the homodimer data were used and the K_D s for the heterodimeric reactions were the fitted parameters. In the c-Rel heterodimer anisotropy experiments, three reactions and five species were involved: $*N_{dd} - *N_{dd} \rightleftharpoons 2 *N_{dd}$, $cRel - cRel \rightleftharpoons 2 cRel$, and

$*N_{dd} - cRel \rightleftharpoons *N_{dd} + cRel$. Again, the K_D s for the RelA or p50 homodimers were obtained from the previous fit of the homodimer data. The K_D for the cRel homodimer equilibrium was tested with a range from 0.1 to 1000 nM and found to have little effect on the fitting result. The K_D for the heterodimeric reaction was the fitted parameter. The concentrations of $*N_{dd} - *N_{dd}$, $*RelA_{dd} - *RelA_{dd}$, and $*RelA_{dd} - RelA$ were found to be negligible compared to $*N_{dd} - N$, $*RelA_{dd} - p50$, and $*N_{dd} - cRel$ in numerical calculations and thus did not contribute to the observed anisotropy change.

Numerical solutions for the I κ B binding equilibria could not be numerically solved. Instead, we include SPR data performed as described previously (21).

Ordinary differential equation modeling.

Dimerization of NF κ B and binding of I κ Bs to the dimers were expressed in rate equations. Kinetics and equilibrium concentrations of NF κ B subunits, dimers, and κ B-complexes were calculated by numerically solving the rate equations using the ordinary differential equation solver ode15s in MATLAB (version R2016a). Initial concentrations were determined from quantitative immunoblots of each NF κ B subunit and I κ B proteins in MEF cells. The association rate constants were initially set to be $10^9 \text{ M}^{-1} \text{ s}^{-1}$ for all association processes as was done previously (3), however, in some cases a range from fast ($10^9 \text{ M}^{-1} \text{ s}^{-1}$) to slower association rates were used. The dissociation rate constants were calculated from the equilibrium constants (K_D) determined by anisotropy experiments and the association rate constants. MATLAB codes are available upon request. We emphasize that the kinetic modeling was only used to determine the abundance of each species once equilibrium was achieved.

Results

Binding affinity of RelA homodimer.

First, the affinity of the RelA homodimer was measured by mixing different concentrations of full-length RelA_{19–325} with 100 pM *RelA_{dd}. Dimer dissociation and reassociation would allow formation of a *RelA_{dd}-RelA_{19–325} dimer, which would have an increased fluorescence anisotropy due to its larger size (Figure 1A). To measure the K_D , we mixed increasing concentrations (0–100 nM) of RelA_{19–325} with a fixed concentration (100 pM) of *RelA_{dd}. Initially, we measured the steady-state anisotropy of each sample after 12 hr, but we observed a peak of maximum anisotropy at ~20 nM (Figure 1B). We surmised that equilibrium may not yet have been reached in the samples containing higher concentrations of RelA_{19–325}, due to rebinding of RelA_{19–325} monomers rather than exchange with the lower concentration of *RelA_{dd} monomers. Measurements were then performed after 24, 36, and 48 hr. At 48 hr, a typical curve for a binding isotherm was observed, and the K_D was determined to be 45 ± 6 nM (Figure 1C). The fact that the peak in anisotropy was observed at ~20 nM after 12 hr and the final K_D was numerically solved to be 45 nM, was suggestive that at the later timepoints, the samples containing higher concentrations of RelA_{19–325} had not yet reached equilibrium due to rebinding of RelA_{19–325} monomers rather than exchange with the lower concentration of *RelA_{dd} monomers. The peak would then be due to the portion of the *RelA_{dd} monomers that had bound to a RelA_{19–325}.

Binding affinity of RelA-p50 heterodimer.

After ascertaining the binding affinity for the RelA homodimer, a similar approach was used to measure the binding affinity of the RelA-p50 heterodimer. In this case, the RelA_{19–325}-p50_{39–363} was mixed with *RelA_{dd} (Figure 2A). Again, after 12 hrs, a peak was observed in the steady-state anisotropy curve, but at a much lower concentration of ~300 pM (Figure 2B). After 48 hr, the binding isotherm could be fit and the K_D was 270 ± 20 pM, indicating much higher affinity for the RelA-p50 heterodimer (Table 1). While it was possible that some *RelA_{dd}-RelA_{19–325} formed, over time this dimer would dissociate due to its weaker binding affinity. The strikingly higher affinity indicated formation of the *RelA_{dd}-p50_{39–363} heterodimer.

Binding affinity of p50 homodimer, RelA-cRel, and p50-cRel heterodimers.

To measure the affinity of the p50 homodimer, *p50_{dd} was mixed with the p50_{39–363} homodimer. Again, after 12 hr, a curve with a peak at ~400 pM was observed and after 48 hr, the binding curve was obtained yielding a K_D of 540 ± 60 pM (Figure 3A). By mixing *RelA_{dd} with cRel_{1–325}, we measured a K_D for the RelA-cRel heterodimer of 5.4 ± 0.9 nM (Figure 3B) and by mixing *p50_{dd} with cRel_{1–325} we measured a K_D for this heterodimer of 3.0 ± 0.3 nM (Figure 3C). All binding affinities are summarized in Table 1.

Binding affinity of I κ B α , I κ B β , and I κ B ϵ to NF κ B homo- and heterodimers.

To measure the affinities of the I κ Bs with the various NF κ Bs, a modification of the fluorescence anisotropy assay was performed in which one subunit of a full-length NF κ B was labeled with Oregon green, and the resulting dimer was held at a low fixed

concentration. Increasing concentrations of each I κ B were added and allowed to reach equilibrium. As before, the NF κ B- I κ B complex tumbled more slowly than the NF κ B alone and the steady-state anisotropy increased as binding saturated. Binding of *RelA1g-321 homodimer to each I κ B showed that I κ B α bound with a K_D of 47 ± 6 pM. For comparison the binding affinity measured by SPR was 54 ± 8 pM. I κ B β bound with a K_D of 25 ± 3 pM, and I κ B ϵ bound with a K_D of 170 ± 10 pM (Figure 4A, Table 1). Thus, RelA homodimer slightly prefers I κ B β over I κ B α , and is strongly inhibited (all binding affinities are in the pM range) by all three I κ Bs.

I κ B α bound to RelA homodimer dimerization domain, *RelA₁₉₀₋₃₂₅-RelA₁₉₀₋₃₂₅ with a K_D of 280 ± 31 pM, showing that the N-terminal domains contribute about 6-fold to the I κ B α binding affinity similar to what was previously reported (21). I κ B β bound to the dimerization domain construct, *RelA₁₉₀₋₃₂₅ with a K_D of 72 ± 6 pM, a difference of three-fold (Figure 4B).

RelA₁₉₋₃₂₅-p50 heterodimer gave a K_D for I κ B α of 42 ± 9 pM, very similar to the value of 39 pM measure by SPR and reported previously (21), a K_D for I κ B β of 890 ± 160 pM, again similar to that measured by SPR of 1100 ± 50 pM. RelA₁₉₋₃₂₅-p50 heterodimer binds to I κ B ϵ with a K_D of 800 ± 110 pM (Figure 4C, Table 1). Thus, the RelA-p50 heterodimer dramatically prefers I κ B α . The binding affinity of I κ B α was within error the same for RelA homodimers (47 pM) as for RelA-p50 heterodimers (42 pM). I κ B ϵ did not bind particularly tightly to either the RelA homodimer or the RelA-p50 heterodimer. We did not assay p50 homodimer for binding to I κ Bs because previous reports indicated that p50 homodimer binds weakly, if at all, to I κ B α (22).

Two NF κ B dimers containing cRel were also characterized for their binding preferences among the I κ Bs. Dimers containing cRel bound most tightly to I κ B ϵ . RelA-cRel, p50-cRel, and cRel homodimer bound to I κ B ϵ with affinities of 98 ± 5 pM, 60 ± 10 pM, and 100 ± 8 pM, respectively (Figure 4D, 4E, Table 1).

Finally, we also mixed *RelA₁₉₋₃₂₁ with RelA₁₉₀₋₃₂₁-p50-I κ B α to see if we could observe dimer exchange when the dimer was bound to I κ B α . No exchange was observed over 72 hours. These results strongly suggest that no free monomer of RelA would be present in the cytoplasm because once an NF κ B dimer was bound to its preferred I κ B, dimer exchange would not occur on a relevant time scale.

Ordinary Differential Equation modeling of subunit exchange.

Our experimental results that showed both RelA-p50 dimerization affinity and I κ B α binding affinity in the pM range suggested subunit exchange may be limited, especially in the presence of I κ B α . To investigate the rates of subunit exchange of RelA-p50 and how I κ B α may affect it, we reasoned that RelA-p50 can re-equilibrate to form two corresponding homodimers. The faster the two subunits in a dimer dissociate and re-associate, the faster they can exchange and form other dimers. Association and dissociation rate constants are connected by the K_D we measured but their values are unknown. Generally, protein association rate constants fall in a wide range from $10^4 \text{ M}^{-1}\text{s}^{-1}$ to $10^9 \text{ M}^{-1}\text{s}^{-1}$ (23). Given

rate constants and initial concentrations, the kinetic process of subunit exchange can be simulated by numerically solving the following ordinary differential equations (ODEs).

$$\begin{aligned}\frac{d}{dt}[A] &= -2k_{a1}[A]^2 + 2kd_1[A:A] - k_{a2}[A][50] + k_{d2}[A:50] \\ \frac{d}{dt}[50] &= -k_{a2}[A][50] + kd_2[A:50] - 2k_{a3}[50]^2 + 2k_{d3}[50:50] \\ \frac{d}{dt}[A:A] &= k_{a1}[A]^2 - kd_1[A:A] \\ \frac{d}{dt}[A:50] &= k_{a2}[A][50] - kd_2[A:50] \\ \frac{d}{dt}[50:50] &= k_{a3}[50]^2 - kd_3[50:50]\end{aligned}$$

We first tested whether subunit exchange could occur in cells, where the estimated amounts of RelA, p50, and IκBα are equal, approximately 350 nM (Supplementary Table 6, Tsui et al, 2015 (17)). As has been done previously (3), the maximal NFκB subunit association rate was initially set to the diffusion limit $k_{a1} = k_{a2} = k_{a3} = 10^9 \text{M}^{-1}\text{s}^{-1}$. The resulting dissociation rates from the measured K_D s of each dimer were calculated (Table 1); $k_{d1} = 18.6 \text{s}^{-1}$; $k_{d2} = 0.3 \text{s}^{-1}$; $k_{d3} = 0.4 \text{s}^{-1}$. The model predicted that under these conditions, RelA-p50 can re-equilibrate and form the two homodimers within only 5 seconds (Figure 5A). If the association rates were 100-fold slower, the dimers would still re-equilibrate in 5 min. Even if the association rates were $10^5 \text{M}^{-1}\text{s}^{-1}$, the dimers would re-equilibrate in 5 hr.

We next tested whether such fast subunit exchange would be affected by IκBα, which is always present in resting cells to keep NFκB in the cytoplasm and inactive. Rate equations including IκBα binding to RelA-p50 were added to the ODE model (Supplementary note 1). The result showed that, starting with RelA-p50-IκBα, subunits never exchange over periods of at least 24 hrs. (Figure 5B). Because of the high affinity of IκBα binding to RelA-p50, even when maximal association rates are assumed, dimer exchange of RelA-p50 is totally blocked by IκBα.

Ordinary Differential Equation modeling of *in vivo* half-life.

Given the widely varying reported affinities of IκBα binding to RelA-p50 from 39 pM (determined by SPR (21) and 42 pM in this work to 1 nM (determined by gel shift (3, 17, 18)), we decided to test whether the ~40 pM affinity could be consistent with the intracellular half-life of RelA-p50-IκBα. Free IκBα degrades rapidly *in vivo* with a short half-life of 5 – 10 minutes, while NFκB-bound IκBα is stable with a half-life longer than 48 hours (11). The concentration change of RelA-p50-IκBα was expressed with the simple rate equations:

$$\begin{aligned}\frac{d}{dt}[A:50] &= -k_a[A:50][\alpha] + k_d[A:50:\alpha] \\ \frac{d}{dt}[\alpha] &= -k_a[A:50][\alpha] + k_d[A:50:\alpha] - k[\alpha] \\ \frac{d}{dt}[A:50:\alpha] &= k_a[A:50][\alpha] - k_d[A:50:\alpha]\end{aligned}$$

The degradation rate constant of free I κ B α , k , is 0.002 s^{-1} (11). We tested both limiting cases of fast and slow association between RelA-p50 and I κ B α . The association rate constant, k_a , was set to be $10^9\text{ M}^{-1}\text{ s}^{-1}$ for the fast case and $10^4\text{ M}^{-1}\text{ s}^{-1}$ for the slow case; the dissociation rate constant k_d was calculated from $K_D k_a$. Our results showed that in both cases the 40 pM affinity greatly stabilizes the protein complex and explains its > 48 hour *in vivo* half-life. If IWe tested both limiting cases of fast and slow association between RelA-p50 and I κ B α bound to RelA-p50 with an affinity of only 1 nM, a substantial portion of free I κ B α was present, and its rapid degradation would drive a decrease in the RelA-p50-I κ B α concentration (Figure 6A, B).

Ordinary Differential Equation modeling of dimer population shifts caused by I κ B preferential binding.

Our anisotropy experiments showed that each I κ B has a binding affinity in the pM range for at least one NF κ B dimer. I κ B α binds preferentially to RelA-p50 and RelA homodimer; I κ B β binds preferentially to RelA-RelA; κ Be binds preferentially to c-Rel containing dimers. To investigate how I κ B preferential binding can shift the equilibrium populations of NF κ B dimers, we performed ODE modeling (Supplementary note 2). In this model, we considered the simplest case where the dominant molecules are RelA, p50, I κ B α and I κ B β , as in mouse embryonic fibroblasts. Our results showed that, in the absence of I κ B α , I κ B β can shift the equilibrium population from RelA-p50 to RelA-RelA-I κ B β (Figure 7), consistent with previous experimental data (17). The preference of I κ B β toward RelA-RelA stabilizes this dimer despite the weaker dimerization affinity of RelA-RelA (45 nM) compared to that of RelA-p50 (270 pM). Interestingly, in the presence of I κ B α , I κ B β is not able to shift dimer equilibrium population, because all RelA-p50 is bound tightly to I κ B α and does not undergo dimer exchange.

Discussion

Most studies of NF κ B monitor fluorescently labeled RelA, but RelA can exist in homodimers or RelA-p50 or RelA-cRel heterodimers, and p50-cRel would not be observed. Each NF κ B dimer has been shown to have distinct functions, with, for example, RelA-p50 being important in inflammation and p50-cRel being important in cancer (24). It therefore seemed important to establish the dimer binding affinities so that complete systems models of all dimers and their inhibitors could be generated, and predictions could be made about when dimer exchange might occur allowing an understanding of how different dimers prevail in different cell types. We developed a fluorescence anisotropy method to measure the dimer equilibrium binding affinities under the lower concentration expected in cells. By mixing and matching labeled and unlabeled dimers with and without the DNA-binding domains, we measured the K_D of each dimer formation. Our results showed that the RelA-p50 dimer has the highest affinity, with a K_D of 270 pM. The p50 homodimer binds nearly as tightly with a K_D of 540 pM. Dimers containing cRel have affinities in the low nM range, and the RelA homodimer is weakest, with an affinity of 45 nM. We note that these dimer binding affinities are much stronger than previously reported based on gel-shift and analytical ultracentrifugation experiments, although the order of affinities is the same as previously reported. Why does it matter that the dimerization affinities are stronger than

have been used in previous models? First, because strong dimer affinities imply that essentially no NF κ B monomers will be present in the cell. Even the weakest RelA homodimer would have only 2.2% of the molecules in monomeric form at cellular concentrations (17). This result should put to rest any further speculation that monomeric NF κ B may be present and functional in cells.

Earlier reports of the binding affinity of I κ B α for RelA-p50 used gel-shift assays and measured a K_D of 1nM. We reported a binding affinity of 39 pM (21), which was more consistent with the long intracellular half-life of this complex, but many in the field still deferred to the earlier report. Here, we not only re-confirm the picomolar binding affinity of I κ B α for RelA-p50, we also report picomolar binding affinities for other I κ B-NF κ B complexes. This means that in the cytoplasm of resting cells, all NF κ B dimers will be bound to inhibitors, and essentially no dimer exchange is expected to occur unless one of the I κ Bs is knocked-out or degraded. Importantly, dimer stabilization by its preferred I κ B provides a simple and precise explanation for the short half-lives of both I κ Bs and NF κ Bs in the absence of their binding partner, but long half-lives when bound. Indeed, the melting temperature of the RelA-p50-I κ B α complex was at least 30 °C higher than that of NF κ B alone (25) and 40 °C higher than I κ B α alone (26).

We found that in most cases, each dimer has a preferred inhibitor; RelA homodimer prefers I κ B β , cRel dimers prefer I κ B ϵ , and RelA-p50 prefers I κ B α . Interestingly, this preferential binding correlates with which NF κ B induces transcription of which inhibitor genes. RelA is required for induction of the I κ B α gene whereas cRel induces only the I κ B ϵ gene (9).

The only exception to exclusivity is that I κ B α binds almost as tightly to RelA homodimers as to RelA-p50 heterodimers. This is a very interesting result in light of our observation that during stimulation, I κ B α is degraded releasing the inhibition of RelA-p50, which then enters the nucleus and activates transcription of new I κ B α . This I κ B α then enters the nucleus and “strips” NF κ B off the DNA and the NF κ B-I κ B α complex is rapidly exported from the nucleus (14). Single cell imaging revealed that nearly all the RelA was rapidly exported from the nucleus, and now we know that is because when I κ B α enters the nucleus, it will remove BOTH RelA-p50 heterodimers and RelA homodimers. If I κ B β is already present in the nucleus, as has been shown previously(27), our results support the idea that it would preferentially capture RelA homodimers upon stimulation, but only until the newly synthesized I κ B α enters the nucleus, at which point all of the DNA-bound RelA-containing dimers would be stripped off the DNA by I κ B α and exported out of the nucleus. Finally, it was reported recently that cRel-containing dimers sometimes replace RelA containing dimers at kB site-containing promoters and promote transcription at later times (28). Our results suggest that this is due to the preferential molecular stripping of RelA-containing dimers by I κ B α leaving the cRel-containing dimers behind in the nucleus to continue transcription of late-onset genes.

Protein Accession Uniprot IDs

P105 (from which p50 is generated) P25799

RelA Q04207

cRel P15307
IkBa P25963
IkBβ Q15653
IkBε O54910

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Fluorescence anisotropy measurement of RelA homodimer binding affinity. A) Schematic diagram of the RelA subunits that were mixed and equilibrated in the experiment. B) Mixing of RelA_{19–325} homodimer with *RelAdd (100 pM) showed peak of maximum anisotropy at ~20 nM after 12 hr of incubation. C) After 48 hr, the binding curve could be fit to a hyperbolic binding isotherm and the K_D was determined to be 45 ± 6 nM.

Figure 2.

Fluorescence anisotropy measurement of RelA₁₉₋₃₂₅-p50₃₉₋₃₆₃ heterodimer binding affinity.

A) Schematic diagram of the RelA-p50 subunits that were mixed and equilibrated in the experiment. B) RelA₁₉₋₃₂₅-p50₃₉₋₃₆₃ was mixed with *RelA_{dd} (100 pM) and after 12 hrs, a peak in the plot of anisotropy vs concentration was observed at 300 pM. C) The binding curve obtained after 48 hr could be fit to determine the K_D was 270 ± 20 pM.

Figure 3.

Fluorescence anisotropy measurement of dimer affinities for p50- and cRel - containing dimers. A) By mixing *p50dd (100 pM) with p50₃₉₋₃₆₃, a K_D of 540 ± 60 pM was determined for the p50 homodimer. B) By mixing *RelA_{dd} (100 pM) with cRel₁₋₃₂₅, a K_D of 5.4 ± 0.9 nM was determined. C) By mixing *p50dd (100 pM) with cRel₁₋₃₂₅, a K_D for this heterodimer of 3.0 ± 0.3 nM was determined.

Figure 4.

Binding affinities of IκBs for NFκB dimers. (A) Schematic diagram showing how the experiments for measuring IκB binding were performed. (B) Binding of the *RelA_{FL} homodimer (10 pM) to each IκB showed that IκBα (black circles) bound with a K_D of 47 ± 6 pM, IκBβ (blue circles) bound with a K_D of 25 ± 3 pM, and IκBε (green circles) bound with a K_D of 170 ± 10 pM. (C) Binding of *RelA_{dd} to each IκB showed that IκBα bound to the dimerization domain construct with a K_D of 280 ± 31 pM and IκBβ bound with a K_D of 72 ± 6 pM. (D) Binding of the *RelA_{FL}-p50 heterodimer (500 pM) gave a K_D for IκBα of 42 ± 9 pM, a K_D for IκBβ of 890 ± 160 pM, and a K_D for IκBε of 800 ± 110 pM. (E) Binding of the *RelA_{FL}-cRel heterodimer (5 nM) gave a K_D for IκBα of 500 ± 90 pM, a K_D for IκBβ of 460 ± 50 pM, and a K_D for IκBε of 98 ± 5 pM. (F) *p50_{FL}-cRel (5 nM) bound to IκBα with a K_D of 440 ± 50 pM, to IκBβ with a K_D of 920 ± 150 pM, and to IκBε with a K_D of 60 ± 10 pM.

Figure 5.

Ordinary differential equation modeling shows that RelA-p50 can only undergo subunit exchange when free from IκBα. A) In the absence of IκBα, RelA-p50 can re-equilibrate to form RelA-RelA and p50-p50 homodimers. At equilibrium, the dominant species is RelA-p50 due to its high dimerization affinity. B) When bound to IκBα, RelA-p50 does not undergo subunit exchange.

Figure 6.

Ordinary differential equation modeling shows that the long *in vivo* half-life of NFκB bound IκBα can only be explained by tight binding (42 pM). (A) If the NFκB-IκBα association rate were diffusion controlled ($10^9 \text{ M}^{-1}\text{s}^{-1}$), the 42 pM binding affinity shows that the half-life of bound IκBα is longer than 24 hrs, consistent with *in vivo* observations. (B) If the NFκB-IκBα association rate were slower ($10^4 \text{ M}^{-1}\text{s}^{-1}$), the half-life of bound IκBα is even slower, but in both cases the 1 nM binding affinity results in dissociation and subsequent degradation of IκBα.

Figure 7.

Ordinary differential equation modeling of the population shift upon increasing IκBβ concentration. In the absence of IκBα and of IκBβ, most of the RelA is bound to p50 (light blue) although some is also present as RelA homodimer (light green). As the concentration of IκBβ increases from 0 to 80 nM, the equilibrium population of free RelA-p50 (light blue), shifts towards IκBβ-bound RelA-p50, denoted A:50:β (dark blue). As the concentration of IκBβ increases, the population of free RelA homodimer is almost completely depleted and The IκBβ bound RelA homodimer (denoted A:A:β) increases over that of A:50:β due to the preferential binding of IκBβ to the RelA homodimer. However, in the presence of IκBα, (right-most bar) RelA-p50 is tightly bound to IκBα (grey) and thus the population shift is blocked.

Table 1.

Binding constants of NFκB dimers

NFκB	Dimer partner	K _D (pM)	IκBα	IκBβ	IκBε (pM)
RelA	RelA	45,000 +/- 6000	47 +/- 6 54 +/- 8 (SPR)	25 +/- 3 70 +/- 4 (SPR)	170 +/- 10
	p50	270 +/- 20	42 +/- 9 39 +/- 3 (SPR)	890 +/- 160 1100 +/- 50 (SPR)	800 +/- 110
	cRel	5400 +/- 900	500 +/- 90	460 +/- 50	98 +/- 5
p50	p50	540 +/- 60	ND	ND	810 +/- 30
	cRel	3000 +/- 300	440 +/- 50	920 +/- 150	60 +/- 10
cRel	cRel	ND	ND	ND	100 +/- 8