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Probing the Solution Structure of IκB Kinase (IKK) Subunit γ and Its Interaction with Kaposi Sarcoma-associated Herpes Virus Flice-interacting Protein and IKK Subunit β by EPR Spectroscopy^{*[5]}

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Background: The IκB kinase subunit γ (IKKγ) is an essential modulator of the IKK signalosome whose solution structure is unsolved.

Results: IKKγ is a parallel coiled-coil comprising two registers accommodated by a twist.

Conclusion: The response of IKKγ to binding of signaling partners is twisting and stiffening, rather than major rearrangement.

Significance: Viral activation of IKKγ exploits the structural motif of the twist.

Viral flice-interacting protein (vFLIP), encoded by the oncogenic Kaposi sarcoma-associated herpes virus (KSHV), constitutively activates the canonical nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) pathway. This is achieved through subversion of the IκB kinase (IKK) complex (or signalosome), which involves a physical interaction between vFLIP and the modulatory subunit IKKγ. Although this interaction has been examined both *in vivo* and *in vitro*, the mechanism by which vFLIP activates the kinase remains to be determined. Because IKKγ functions as a scaffold, recruiting both vFLIP and the IKKα/β subunits, it has been proposed that binding of vFLIP

could trigger a structural rearrangement in IKKγ conducive to activation. To investigate this hypothesis we engineered a series of mutants along the length of the IKKγ molecule that could be individually modified with nitroxide spin labels. Subsequent distance measurements using electron paramagnetic resonance spectroscopy combined with molecular modeling and molecular dynamics simulations revealed that IKKγ is a parallel coiled-coil whose response to binding of vFLIP or IKKβ is localized twisting/stiffening and not large-scale rearrangements. The coiled-coil comprises N- and C-terminal regions with distinct registers accommodated by a twist: this structural motif is exploited by vFLIP, allowing it to bind and subsequently activate the NF-κB pathway. *In vivo* assays confirm that NF-κB activation by vFLIP only requires the N-terminal region up to the transition between the registers, which is located directly C-terminal of the vFLIP binding site.

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[5] This article contains supplemental Table 1 and supplemental Movie 1.

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Activation of the canonical nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)⁷ transcriptional pathway occurs in response to a wide variety of cellular stimuli, including cell differentiation, infection, and stress responses (1). It is normally tightly regulated since constitutive activation can lead to prolonged cellular survival and the production of inflammatory

⁷ The abbreviations used are: NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; vFLIP, viral Flice-interacting protein; KSHV, Kaposi sarcoma-associated herpes virus; IKK, IκB kinase; NEMO, NF-κB essential modulator; EPR, electron paramagnetic resonance; MD, molecular dynamics; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factors; TEV, tobacco etch virus; IPTG, isopropyl 1-thio-β-D-galactopyranoside; CVs, column volumes; LV, lentiviral vector; RMSDs, root mean square deviations.

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cytokines both of which have been directly linked to cancer and inflammatory diseases. The pathway converges on a set of NF- κ B transcription factors that in resting cells are localized to the cytoplasm because of their association with inhibitory I κ B proteins (2–4). Liberation of the NF- κ B transcription factors and their transition to the nucleus requires degradation of the I κ Bs that are first phosphorylated and subsequently targeted for proteolysis by the 26S proteasome following Lys-48 ubiquitination. Phosphorylation is facilitated by I κ B kinase (IKK) or signalosome that minimally comprises the kinase subunits IKK α and/or IKK β together with a modulatory element IKK γ (also known as NEMO: NF- κ B essential modulator). This assembly is the target for several viruses since its constitutive activation results in the downstream overproduction of proteins that indirectly promote viral propagation and proliferation through their capacity to prolong cellular survival (5, 6). One such virus is Kaposi sarcoma-associated herpes virus (KSHV) that during its latent phase encodes vFLIP (7). The pathogenicity of vFLIP appears to derive from its capacity to render IKK constitutively active by associating with its modulatory element IKK γ (8, 9). This prolonged activation has been directly linked to Kaposi sarcoma (KS) and other KSHV associated malignancies that includes primary effusion lymphoma (PELs) and multicentric Castleman disease where knockdown of vFLIP alone is sufficient to arrest the growth of KS tumors and kill PELs cells (10, 11). The crystal structure of the vFLIP-IKK γ complex (12) has revealed the nature of this interaction at the atomic level, which was later verified *in vivo* (13). However, the mechanism by which vFLIP is able to activate the IKK α and IKK β kinases remained unclear, especially because they associate with the N terminus of IKK γ , which is more than 200 residues from the vFLIP binding site. Furthermore, recent studies have shown that the ability of vFLIP to activate the canonical NF- κ B pathway appears to be independent of up and downstream effectors such as: tumor necrosis factor (TNF) receptor-associated factors (TRAF)-2, TRAF-3, TRAF-6; linear ubiquitin chain assembly complex (LUBAC); and transforming growth factor (TGF)- β -activated kinase 1 (TAK1) that are all essential for the mechanisms utilized by pro-inflammatory cytokines (14).

It has been proposed that vFLIP activation of IKK involves conformational changes within the IKK γ molecule that effectively switches the assembly from an inactive to an active state that would favorably juxtapose IKK α/β for either trans or autophosphorylation (12). Because there is currently neither a crystal structure of full-length IKK γ nor a fragment that encompasses both the kinase and vFLIP binding sites (either alone or in the relevant complexes) to allow direct testing of this hypothesis in terms of both local and global transitions, we used electron paramagnetic resonance (EPR) spectroscopy to determine distances between spin-labeled cysteine residues introduced at intervals along IKK γ . These allowed *in silico* models of IKK γ to be validated, enabling a solution structure of IKK γ to be obtained for the first time. Measurements were also performed in the presence of vFLIP and separately an IKK β fragment comprising the IKK γ binding site to provide readout of any induced conformational changes.

Experimental Procedures

Cloning—Native full-length human IKK γ degrades at the N and C termini prompting the use of a truncated construct, encompassing residues 40–354. Human IKK γ (40–354) was amplified by polymerase chain reaction (PCR) using the plasmid pGEX-KT IKK γ (8). This was performed using the primers 5'-TTTTGGATCCCACCTGCCTTCAG-3' and 5'-TTTTGAATTCCTAGTCCTCGATCCTGGC-3' containing the BamHI and EcoRI (underlined) restriction sites, respectively. The resulting PCR product was cloned into pETM442 vector (details can be found in the supplemental data of Ref. 12) that had previously been digested using the same enzymes. The resulting construct included a tobacco etch virus (TEV) protease cleavable N-terminal 6His-NusA tag directly 5' to the IKK γ insert as an aid to protein solubilization and purification. To prevent further proteolysis, Gln-83 was mutated to alanine. All IKK γ mutants were produced using the QuikChange kit (Agilent). A list of primers is provided (Supplemental Table S1). The IKK γ interaction domain of IKK β (amino acids 644 to 756) was PCR amplified from the pRC-actin IKK β plasmid that incorporated the full human construct using the primers 5'-CACCGTCCGGCTGCAGGAG-3' and 5'-TCATGAGGCTGCTCCAGGC-3'. It was cloned into pET151/D-TOPO[®] (Invitrogen) to provide an N-terminal 6His-tag cleavable by TEV protease. pETM6T1 vFLIP (1–178) from KSHV, also encompassing a TEV protease cleavable N-terminal 6His-NusA-tag, is described elsewhere (12).

Expression—Wild type and mutant pETM442 IKK γ (40–354) and pETM6T1-vFLIP (1–178) were transformed into BL21(DE3)Star (Invitrogen) that harbored the pRARE2 plasmid encoding rare tRNAs (Novagen). pET151 IKK β (644–756) was transformed into Rosetta-gami B (DE3) (Novagen). The cells were grown in 100 ml of 2YT medium (1.6% (w/v) bacto-tryptone (Invitrogen), 1% bacto-yeast extract (Invitrogen) and 0.5% NaCl, adjusted to pH 7.2) containing chloramphenicol (35 μ g ml⁻¹) and supplemented with either ampicillin (100 μ g ml⁻¹) for IKK γ , kanamycin (24 μ g ml⁻¹) for vFLIP, or kanamycin (24 μ g ml⁻¹), ampicillin (100 μ g ml⁻¹) and tetracyclin (12.5 μ g ml⁻¹) for IKK β , and grown at 37 °C overnight. 1 in 100 dilutions of overnight culture were inoculated into 2YT medium and grown to an OD₆₀₀ of 1.0 at 37 °C before induction with 1 mM isopropyl 1-thio- β -D-galactopyranoside (IPTG) for IKK γ and vFLIP or 0.1 mM IPTG for IKK β , and allowed to grow at: 30 °C for 3 h (IKK γ); 16 °C overnight (vFLIP); or 20 °C overnight (IKK β). The cells were then harvested by centrifugation, washed with buffer A (200 mM NaCl, 25 mM Tris, pH 8.5) and stored at -70 °C.

Protein Purification—Cell extracts were re-suspended in buffer A supplemented with DNase I (10 μ g ml⁻¹ final concentration) and EDTA-free protease inhibitor mixture tablets (Roche). After sonication on ice, lysates were clarified by centrifugation (46,000 \times g for 1 h at 4 °C) and supernatants filtered through a 0.45 μ m filter prior to loading onto a 5 ml of His-Trap[™] FF column (GE-Healthcare). The column was washed with 20 column volumes (CVs) of buffer A containing 50 mM imidazole and the protein eluted with 5 CVs of buffer A containing 500 mM imidazole. The tags were cleaved with TEV

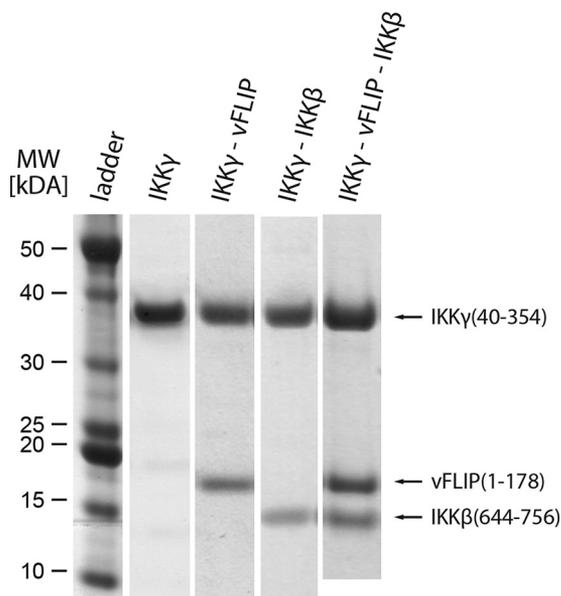


FIGURE 1. SDS-PAGE Coomassie-stained gels of the final size-exclusion profiles obtained for: IKK γ ; IKK γ -vFLIP complex; IKK γ -IKK β complex; IKK γ -vFLIP-IKK β ternary complex of the 56–95 double mutant.

protease and overnight dialysis against buffer A supplemented with 1 mM DTT and 0.5 mM EDTA for vFLIP and IKK β . For IKK γ , the buffer pH was adjusted to 7.5 and maintained at this pH for the following anion exchange step. Cleaved protein solutions were diluted to 50 mM NaCl before loading onto a 5 ml HiTrap Q FF column (GE-Healthcare) and the proteins eluted with a 50 to 500 mM NaCl linear gradient (20 CVs). IKK γ was then spin-labeled as described in the following section. IKK β and vFLIP were dialyzed against 25 mM Tris, pH 7.5, 200 mM NaCl, 10% glycerol, and concentrated to 2 mg ml⁻¹ prior to addition of spin-labeled IKK γ .

Spin Labeling of IKK γ and Purification of Protein Complexes—IKK γ was incubated with a 20-fold excess of 3-(2-iodoacetamido)-proxyl (Aldrich) spin label overnight at 4 °C in the dark. The sample was concentrated to less than 1 ml using a 30 kDa cut-off Vivaspine centrifugal concentrator (Vivascience) before removal of free spin label by size exclusion chromatography using a Superdex 200 HR 10/60 column, pre-equilibrated with 25 mM Tris, pH 7.5, 200 mM NaCl, 10% glycerol. Prior to spectroscopic analysis, the IKK γ buffer was exchanged through concentration and dilution into one in which H₂O was replaced by D₂O. Alternatively, IKK γ was incubated with either IKK β or vFLIP at 4 °C overnight for complex formation. These were then purified by size exclusion chromatography as described for IKK γ alone. SDS-PAGE Coomassie-stained of final size-exclusion profiles following purification for the 56–95 double mutant are depicted in Fig. 1. All other constructs gave similar results. The complexes were subsequently buffer exchanged into D₂O buffer before analysis. The stability of each labeled mutant was assessed by comparing its elution volume on a size exclusion chromatography to that of wild type IKK γ : all had similar profiles. The final concentration of samples for spectroscopic analysis was in the 25–100 μ M range. These gave labeling efficiencies of above 70% as determined by continuous-wave EPR.

EPR Spectroscopy—EPR-based distance measurements were performed at 50 K after flash freezing the samples in liquid nitrogen on an ELEXSYS E580 spectrometer (Bruker) operating at 9.6 GHz equipped with an ER 4118 X-MD5 resonator, a CF935 continuous flow cryostat (Oxford Instruments) and ITC503 temperature controller. The 4-pulse Double Electron-Electron Resonance sequence (15) used was $\pi/2(\nu_{obs})-\tau_1-\pi(\nu_{obs})-t-\pi(\nu_{pump})-(\tau_1+\tau_2-t)-\pi(\nu_{obs})-\tau_2-echo$, where the observer pulse length was 16 ns for $\pi/2$ and 32 ns for π pulses. The pump pulse length was 12 ns. The long interpulse delay (τ_2) was 3500–4500 ns for the single mutants and 4000–5500 ns for the double mutants. All other parameters were according to Ref. 15 with $\tau_1 = 400$ ns and $\Delta\tau_1 = 56$ ns. Data points were collected in 8 ns time steps. The total measurement time for each sample was in the range of 8 to 36 h. The spectra were analyzed using the program DeerAnalysis2011 (16). The background was corrected by a homogeneous three-dimensional fit and the distance distributions evaluated by Tikhonov regularization.

Pairs of residues were chosen so that the intra-pair distance was 2–3 nm, and the inter-pair distance was 4–6 nm. This choice ensured that the r^{-3} dependence of the dipolar interaction, whereby doubling the distance results in 8-fold lower dipolar frequency, separated the inter-pair from the intra-pair frequency while keeping the longer distance accessible. As illustrated in Fig. 2, in a coiled-coil, the geometry is constrained by the intra-pair distance (designated a), and the inter-pair distance (designated b) and dihedral angle (designated θ) between the vectors joining the pairs. There are two long distances between the labels, which are given by the expression: $\sqrt{a^2/2(1 + \cos\theta) + b^2}$. The two limiting cases are when the vectors joining the pairs are parallel (Fig. 2A) or when the vectors joining the pairs are perpendicular (Fig. 2B). In the former $\theta = 0^\circ$, and the labels form a rectangle with two distinct long distances: corresponding to the sides, b , and the diagonals $\sqrt{a^2 + b^2}$ (Fig. 2C, gray lines). In the latter $\theta = 90^\circ$ and there is a single long distance: $\sqrt{a^2/2 + b^2}$ (Fig. 2C, black lines). As illustrated in Fig. 2D, the shape of the distance distribution is strongly dependent on θ , and hence the geometry of the coiled-coil and changes thereto may be evaluated by EPR spectroscopy.

Note that for several cases, fewer spin pairs were observed for the IKK γ -IKK β or IKK γ -vFLIP complexes; this is apparent from the relative modulation depth of the dipolar evolution. However, such differences can arise from the labeling efficiency varying between the samples, making interpretation ambiguous so we do not pursue it here.

MD Simulations—Coarse-grained MD simulations were performed using the MARTINI force field over 1 μ s (17, 18). In brief, ~ 4 non-hydrogen atoms are modeled as a single coarse-grained particle. Non-bonded Lennard-Jones interactions are based on 4 classes of particles (polar, apolar, non-polar, and charged), which are further divided into up to 5 subtypes. Interactions between different classes and sub-classes of particle are calculated based on an interaction table with 9 distinct interaction types. Furthermore, Lennard-Jones interactions were shifted to zero between 0.9 and 1.2 nm. Electrostatics were treated coulombically with a cut off at 1.2 nm (interactions are shifted to zero between 0 and 1.2 nm). α -helical secondary

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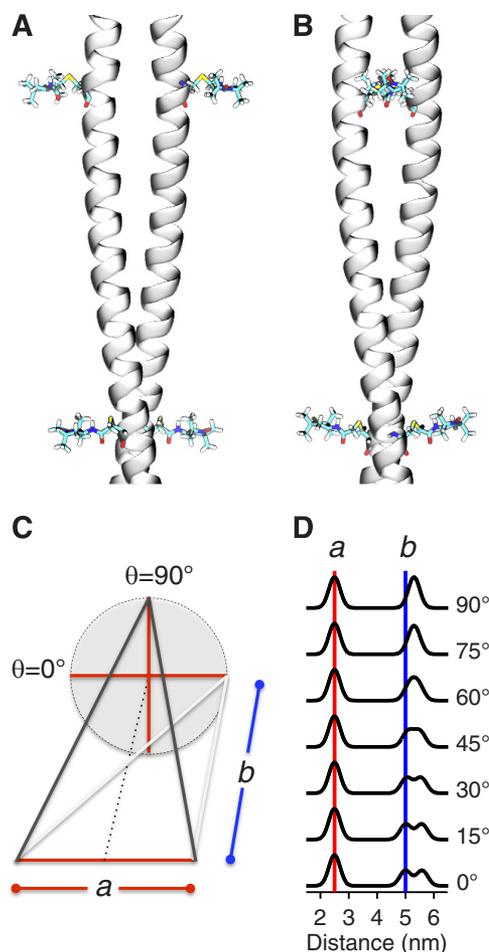


FIGURE 2. Diagram illustrating how the register of the coiled-coil translates into distance distributions between two pairs of nitroxide spin labels. Limiting cases in which (A) all 4 labels are in a plane or (B) the two vectors joining the pairs are perpendicular. C, geometry is constrained by the intra-pair distance (designated a), the inter-pair distance (designated b) and dihedral angle (designated θ) between the vectors joining the pairs. D, illustration of the variation of the distance distribution for two pairs of labels as a function of θ for $a = 2.5$ nm and $b = 5$ nm with Gaussian linewidth of 0.2 nm.

structure was maintained through dihedral angle restraints. Temperature and pressure were coupled at 323 K and 1 bar, respectively using the Berendsen weak coupling algorithm ($T_{\tau} = 1$ ps and $T_p = 10$ ps). Simulations were performed with Gromacs 4.5 (19). This generated an extended simulation trajectory from which 2,500 frames were used as template structures in MODELLER to generate new all-atom models of IKK γ . As each of these structures represents a distinct point in the dynamic ensemble, each of the structures has similar limitations to the original model. To include all of the conformational variability observed across the simulation, distance predictions using a rotamer library approach (20) were made for the labels in each frame and combined to generate a single distribution representing all label positions for all observed backbone bending, across the complete dynamic ensemble. This approach is a variation on serial multiscale modeling approaches (21), which explicitly samples across the entire trajectory rather than the end point alone.

NF- κ B Luciferase Reporter Assays—Reporter assays were performed on 70Z/3 or Jurkat IKK γ -null cells stably reconstituted with IKK γ mutants as previously described (13). Recon-

stituted cells were transduced with a lentiviral vector (LV) encoding an NF- κ B responsive luciferase reporter. 5×10^4 cells were seeded in optical bottom 96-well plate and transduced with a vFLIP encoding LV. 48 h later, 50 μ l of BrightGlo luciferase substrate (Promega) was added to each well and the NF- κ B-induced luminescence was detected using Varioskan Flash multimode reader (Thermo Scientific). Where indicated cells were treated for 6 h with lipopolysaccharide (10 μ g ml $^{-1}$) prior to substrate addition.

Results

Analysis of IKK γ using Single Pairs of Spin Labels—The existing crystal structures of IKK γ fragments (12, 22–25) all exhibit coiled-coil structures (Fig. 3A). Furthermore, 4 different coils prediction algorithms (26–29) suggest that the entire molecule (excluding the C-terminal zinc finger) adopts a parallel coiled-coil conformation concomitant with a switch in helical register close to Val-250. The results from Multicoil2 (28) are depicted in Fig. 3, B–D. To validate the prediction we employed EPR distance measurements; nitroxide spin-labeling of a single cysteine in a homo-dimeric protein such as IKK γ automatically gives rise to a pair of labels, whose separation may be measured by EPR spectroscopy.

IKK γ , however, contains 6 native cysteine residues distributed throughout the sequence. To establish whether their removal (in order to produce mutants containing a single cysteine for spin-labeling studies) would have a deleterious effect on the activity of IKK γ , a mutant involving the full-length protein in which 5 cysteines were mutated to serines (leaving only Cys-167) was constructed and tested *in vivo* using a NF- κ B luciferase reporter assay. Although the levels of NF- κ B activation were reduced relative to wild type IKK γ , the mutant was still able to activate the pathway when stimulated by vFLIP (Fig. 4). In contrast, significant attenuation for this mutant was observed following stimulation using lipopolysaccharide (LPS) indicating that at least some of these residues have a role in cytokine induced activation. Occasionally, it was found that certain mutants containing a single native cysteine (and 5 serines) were unsuitable for EPR studies or were poorly expressed. In these cases, alternative mutants were constructed in which the cysteine was shifted to a neighboring site. Thus, mutants containing a single cysteine at 7 different positions (95, 116, 133, 169, 230, 265, and 347) along the length of the 40–354 IKK γ fragment were constructed, expressed and spin-labeled. The positions of the labels in the sequence are indicated in Fig. 3E (solid circles).

The background-corrected dipolar evolution and derived fits (Fig. 5A, black and red lines, respectively) obtained by Tikhonov regularization all show a damped oscillation that reaches a plateau after several hundred nanoseconds. The corresponding distance distributions (Fig. 5B, red lines) show a single major peak centered below 3 nm. This confirms that IKK γ is a parallel coiled-coil along its entire length consistent with the crystal structures of fragments and prediction algorithms.

A Static Model of IKK γ —An initial model of IKK γ was constructed from a canonical coiled-coil namely Liprin B (Protein Data Bank (PDB) entry 3QH9 (30)). This was extended by iteratively overlaying carbon alpha atoms of the termini, and

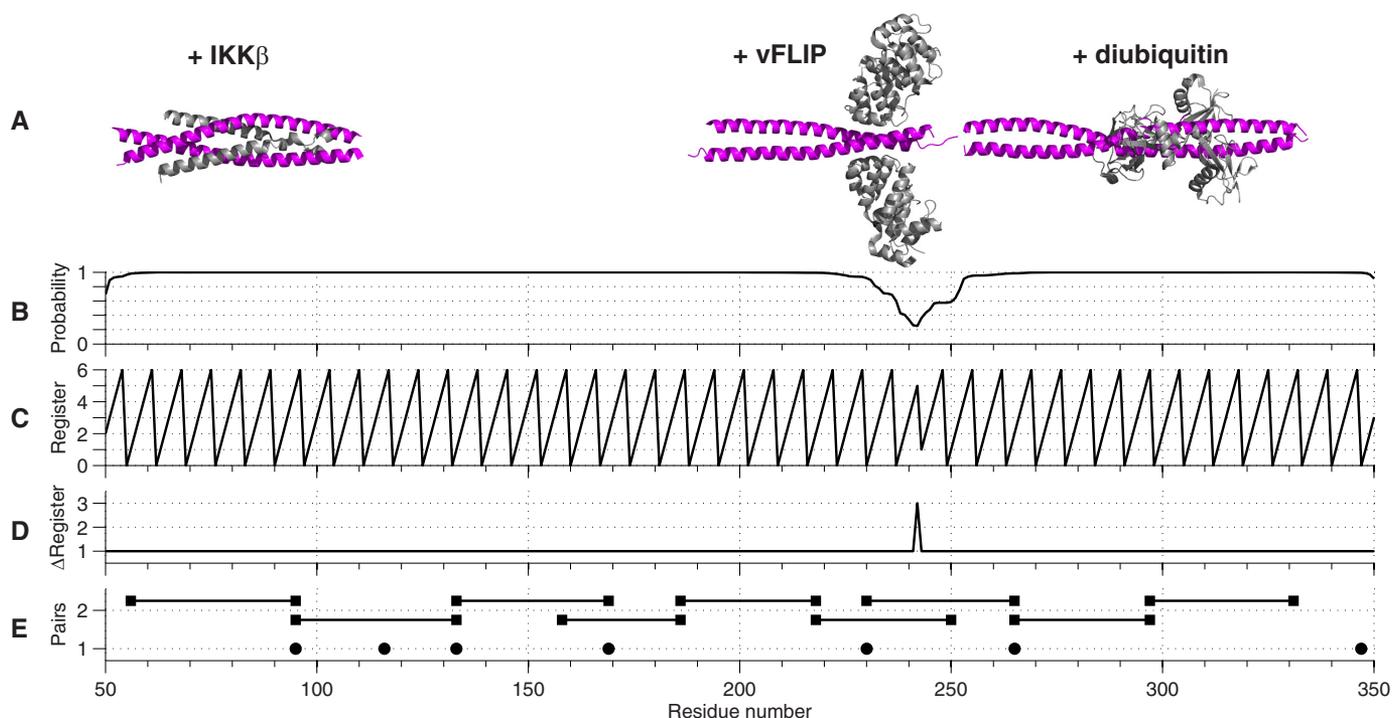


FIGURE 3. *A*, crystal structures of fragments of IKK γ (magenta) in complex with IKK β , vFLIP, and diubiquitin (gray) aligned according to their position in the sequence. *B*, probability of a region adopting a coiled-coil arrangement based on a window of 28 residues predicted from the primary sequence of IKK γ . *C*, predicted position of residues within a coiled-coil based on the heptameric repeat of the motif: each residue has a predicted position from 0–6 and a regular zigzag pattern implies a perfect coiled-coil. *D*, increment of the register of a residue with respect to its predecessor. An increment of +1 implies a perfect coiled-coil. *E*, positions of nitroxide spin labels for singly labeled IKK γ (solid circles) and doubly labeled IKK γ (solid squares joined by solid lines).

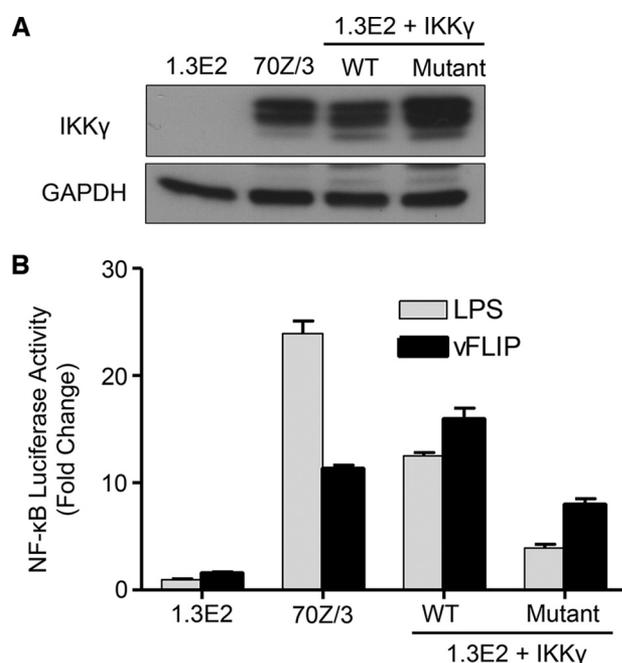


FIGURE 4. **NF- κ B reporter assays for the vFLIP mutant in which all 5 out of 6 cysteines were mutated to serine.** *A*, Western blot analysis of IKK γ expression level in IKK γ knock-out mouse PreB cell line 1.3E2, respective parental cells 70Z/3 and IKK γ wild type or mutant reconstituted 1.3E2 cells. *B*, NF- κ B activation was measured in the above-mentioned cell lines using the BrightGlo Luciferase assay system, 6 h after stimulation with LPS ($10 \mu\text{g ml}^{-1}$) or 48 h after transduction with KSHV vFLIP LV (MOI = 50). Bars represent mean fold induction values \pm S.D.

recording new predicted atom positions. To explore the full heptad pattern of alternative coiled-coil registers, the alignment of the sequence to the template was shifted stepwise by

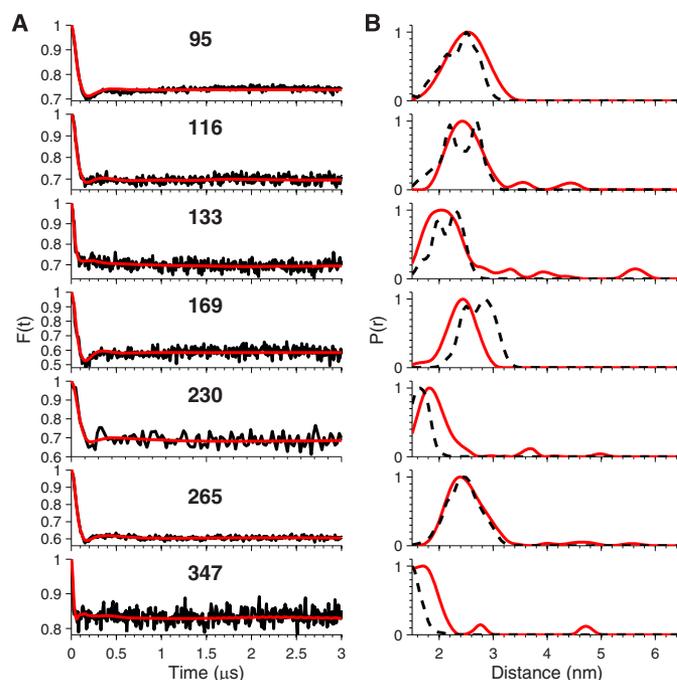


FIGURE 5. **EPR distance measurement on singly-labeled IKK γ .** *A*, background-corrected dipolar evolution (black lines) and their fits (red lines). *B*, distance distributions (red lines) derived from the data in *A* and predicted distance distributions (black dashed lines) derived from the static 3/5 hybrid model.

0–6 residues in MODELLER (31). Distance distributions derived from the 7 models (Fig. 6, blue lines) using a rotamer library approach (20) were then compared with the EPR data (Fig. 6, magenta lines). The cumulative difference between

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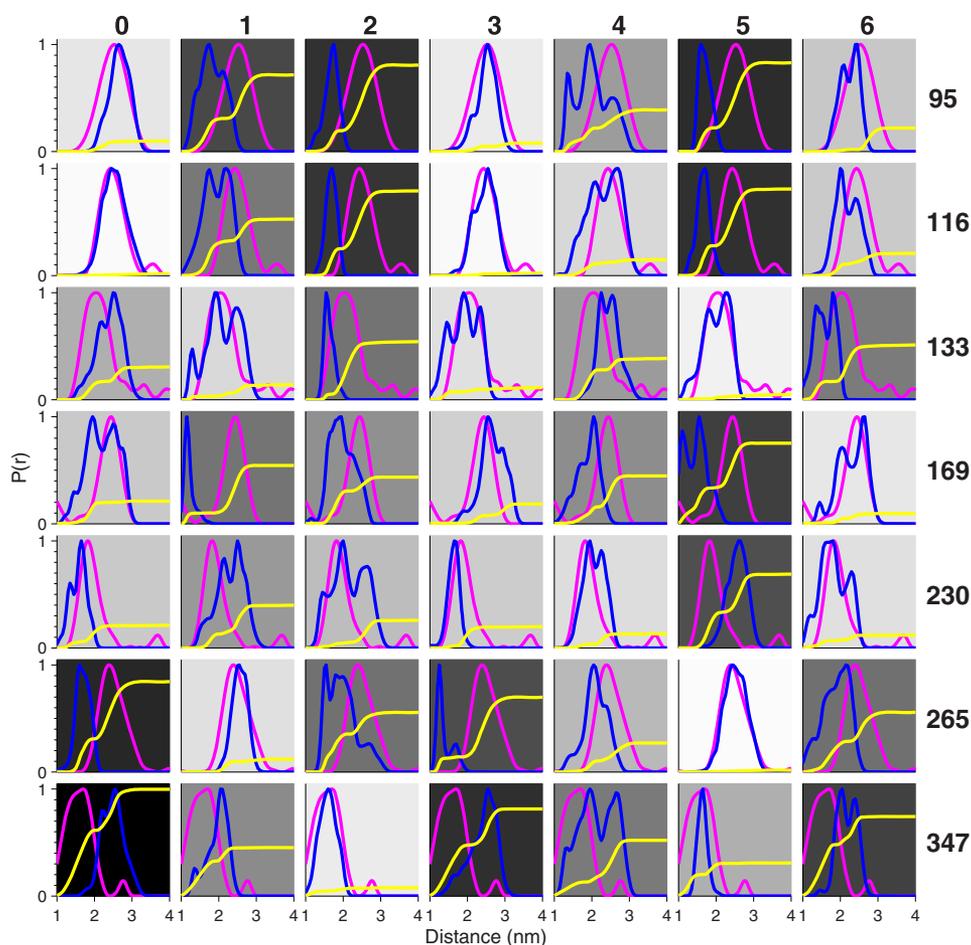


FIGURE 6. Validation of the model structures. Comparison of the distance distributions obtained from the EPR experiments for the singly-labeled IKK γ (magenta lines) with the predicted distance distributions (blue lines) using a rotamer library approach for the 0–6 register models. The cumulative sum of the squares of the differences between the experimental and predicted distributions is used to provide a metric for goodness of fit (yellow lines). The background has been gray-shaded according to the relative final amplitude of the yellow lines: a white background implies perfect match, whereas a black background implies poor agreement.

experiment and model was used as a metric of the (dis-)agreement between them (Fig. 6, yellow lines). To aid visual comparison, the background of each plot has been gray-shaded according to the difference: a white implies perfect agreement; whereas black implies the largest deviation.

Register-3 and, to a slightly less extent, Register-0 gave the best agreement for positions 95, 116, 133, 169, and 230; while Register-5 gave the best agreement for positions 265 and 347. We therefore constructed two hybrid models combining the 0 or 3 and 5 registers for the N and C termini respectively, with the junction of the two registers in the region between amino acid residues Gln-231 and Lys-264. The key difference between the models is the handedness of the twist between different registers; the twist of the 0/5 hybrid is right-handed, while the twist of the 3/5 hybrid is left-handed. Both maintain the hydrophobic interface, while changing the specific angle the helices adopt relative to one another. The predicted distance distributions derived from the 3/5 hybrid gave best agreement with the single mutant EPR data (black dashed lines, Fig. 5B).

An overlay of the 3/5 hybrid with three crystal structures is presented in Fig. 7A. To obtain a metric of the deviation of the 7 models with crystal structures of IKK γ fragments, the root mean square deviations (RMSDs) of the positions of the non-

hydrogenic atoms in these regions were calculated (Fig. 7, B–D). As expected the RMSDs follow a sinusoidal pattern covering two complete oscillations over 7 residues. The IKK γ + IKK β crystal structure (PDB entry 3BRT (22), residues: 50–110) has the closest match with Register-4 followed by Register-0 (Fig. 7B) while the crystal structure of IKK γ + vFLIP (PDB entry 3CLC (12), residues: 195–250) has closest match with Register-3 followed by Register-6 (Fig. 7C). By contrast, crystal structures of the ubiquitin binding region both alone (PDB entry 2ZVN (24), residues: 270–330) and in complex with diubiquitin (PDB entry 3FX0 (23)) and Hiop (PDB entry 4OWF (25), residues: 255–335) have closest matches with Register-2 followed by Register-5 (Fig. 7D). Thus both 0/5 and 3/5 hybrid models that we constructed from the EPR data are consistent with crystal structures giving confidence that we have derived a realistic overall view of the structure of IKK γ in solution.

Analysis of IKK γ Alone and in Complex with vFLIP and IKK β using Two Pairs of Spin Labels—The results presented above do not answer the further question as to whether the coiled-coil does or does not deviate from an extended structure. Hence, to provide a more detailed survey of the IKK γ architecture and investigate any large-scale reorganization of IKK γ involving N- and C-terminal movements following association with vFLIP or

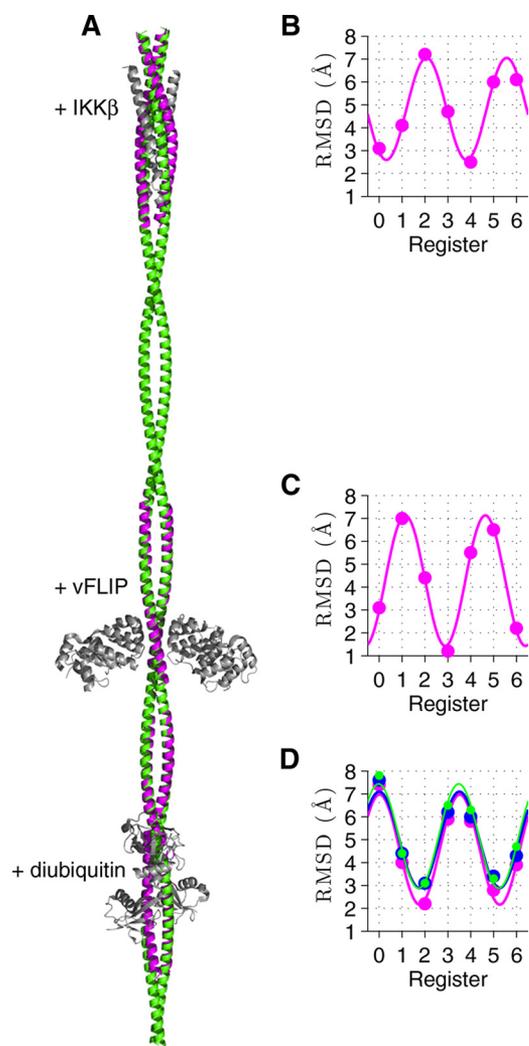


FIGURE 7. Comparison of the 3/5 model of IKK γ with crystal structures. A, 3/5 model (green) is overlaid with crystal structures of IKK γ fragments (magenta) with IKK β , vFLIP, and diubiquitin (gray). Plots of the RMSDs of the positions of the non-hydrogenic atoms in the coiled-coil model structures (Registers 0–6) compared with those in the known crystal structures: (B) IKK γ + IKK β (22); (C) IKK γ + vFLIP (12); (D) IKK γ ubiquitin binding region: apo (23) (blue), with diubiquitin (24) (magenta), and with Hiop (25) (green). The curves are fits to the RMSDs assuming a sinusoidal pattern that completes two oscillations within the heptad repeat.

IKK β , a series of 9 cysteine double mutants (56–95; 95–133; 133–169; 158–186; 186–218; 218–250; 230–265; 265–297; and 297–331) were constructed and spin-labeled. The positions of the pairs in the sequence are given in Fig. 2E (solid squares).

The dipolar evolution of the 9 double mutants was recorded for IKK γ alone (green lines) and in complex with IKK β (blue lines) and vFLIP (magenta). Inspection of the background-corrected dipolar evolution (Fig. 8) reveals that they contain both a high frequency component comparable to those seen in the spectra for pairs of spin labels (Fig. 5A) and a lower frequency component that extends out to several microseconds, indicative of longer distances.

Many of the data sets show small changes in profile upon binding of IKK β or vFLIP, especially at the inflection around 200 ns, whereas the lower frequency component is largely unchanged. These observations are reflected in the corresponding distance distributions (Fig. 8). These exhibit a rich

variety of shapes and all show a major peak with a maximum in the range 2–3 nm as expected for the intra-pair distance and additional peaks in the range 4–6 nm, which arise from the inter-pair distances.

56–95, 95–133, and 133–169, give single sharp long distance peaks at just below 6 nm. This indicates that the pairs of vectors joining the spin labels are close to being perpendicular and that the coiled-coil is well defined in this region. The distributions are only subtly affected by binding of IKK β or vFLIP, although we note that vFLIP induced denaturation of the 95–133 construct for reasons that are not understood.

158–186 has a partially resolved pair of peaks centered at 4.5 nm indicating that the dihedral angle between the pairs of vectors joining the spin labels deviates from 90°. No significant change occurs when IKK β binds, but vFLIP binding induces a broadening of the long distance distribution that may be indicative of twisting.

186–218, 218–250, and 230–265 all show a much broader distribution for the long distances, indicating that not only is the dihedral angle between the pairs of vectors joining spin labels far from 90°, but that the coiled-coil is reasonably flexible in this region. Neither IKK β nor vFLIP have a significant effect on the distance distributions for 186–218 and 218–250, whereas for 230–265 vFLIP binding results in a remarkable sharpening of the intensity at 6 nm (magenta line), indicating that the angle between the pairs of vectors joining the spin labels tends toward 90° and that the coiled-coil has become more rigid in this complex.

265–297 and 297–331 both display a pair of long distance peaks indicating that the dihedral angle between the pairs of vectors joining the spin labels deviates significantly from 90° and that the coiled-coil is more rigid. Only minor broadening effects are observed when IKK β or vFLIP bind.

To summarize, the double mutants provide a picture of IKK γ as an extended coiled-coil that remains largely unchanged upon binding of either IKK β or vFLIP, although the binding of vFLIP stiffens the region spanning 230–265 (where it binds) and twists the region spanning 158–186.

Toward a Dynamic Model of IKK γ —The 3/5 hybrid model derived above gives reasonable agreement with the distance distributions observed with both one and two pairs of spin labels, although the predictions exhibit fine structure absent from the experimental data that is likely to have arisen because the model is static whereas IKK γ is potentially a dynamic molecule that can flex and bend. Hence, we performed a 1 μ s MARTINI (17, 18) coarse-grained MD simulation of the 3/5 hybrid structure in water, with counter-ions that allowed for bending of the structure (Supplemental Movie). This dynamic model results in a better fit to the experimental distance distributions of the doubly-labeled IKK γ (Fig. 8, black dashed lines) supporting our conclusion that the structure is an extended coiled-coil. Additionally, visual inspection of the simulation suggests that the transition between the 3- and 5-registers that we identified as occurring in the region between Gln-231 and Lys-264 gives rise to a twist centered on residues Lys-246 to Ser-248, and that the whole structure appears to hinge about the twist.

Solution Structure of IKK γ

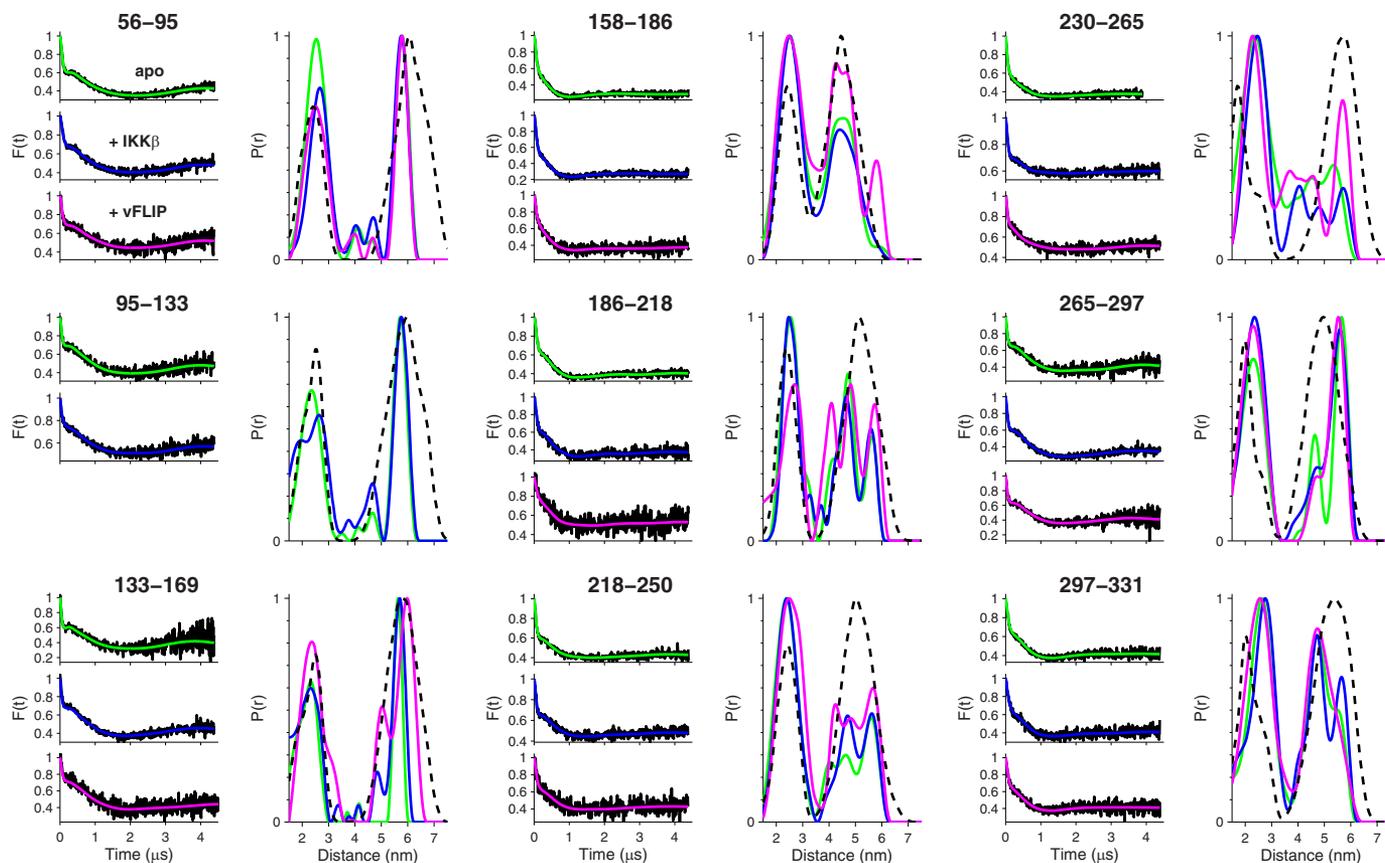


FIGURE 8. EPR distance measurements on doubly-labeled IKK γ , apo and complexed with IKK β and vFLIP. Fits to the baseline-corrected dipolar evolution and corresponding distance distributions for apo IKK γ (green lines) and IKK γ complexed with IKK β (blue lines) and vFLIP (magenta lines). The distance distributions for the doubly labeled IKK γ derived from the coarse-grained MD simulation of the 3/5 hybrid model are shown as a dashed black line.

Activation of IKK γ by vFLIP—We have shown that IKK γ is essentially composed of two regions with different helical registers. The C-terminal region containing the ubiquitin binding region ends just before the vFLIP binding site. To investigate whether its absence would have any impact on the capacity of vFLIP to activate the canonical NF- κ B pathway, IKK γ truncation mutants were generated that terminated at Arg-254 (Δ 254–419) within the discontinuity and at Glu-271 (Δ 271–419) C-terminal to this region. Both mutants induced near wild type levels of activation in a NF- κ B luciferase reporter assay (Fig. 9). This is in contrast to induction with LPS where they were found to be highly defective because of deletion of crucial ubiquitin binding residues C-terminal to Glu-271. Interestingly, a point mutation D242R at the vFLIP binding site within the transition between the registers renders IKK γ constitutively active in the absence of LPS (Fig. 9). Our findings thus confirm that the N-terminal region of IKK γ is alone sufficient for vFLIP-induced activation and are consistent with previous results for an IKK γ -estrogen receptor fusion protein in which IKK γ was truncated at Gly-251 (32), but also illustrate that the observed change in register is functionally important with respect to activation by endogenous cytokines.

Discussion

It has been established that constitutive activation of the canonical NF- κ B pathway by the KSHV is pivotal to viral pathogenesis having been directly linked to KS and the other lym-

phoproliferative disorders. Although key to this process is a physical interaction between virally encoded vFLIP and IKK modulatory subunit IKK γ that has been extensively characterized, it remains unclear how persistent activation is achieved. It has been proposed that IKK γ is held in a configuration that promotes phosphorylation of the kinases (either through autophosphorylation or recruitment of upstream kinases) distinct to that of its unbound state in response to vFLIP binding (12). This mechanism of IKK activation was first put forward for the T-cell leukemia virus oncoprotein TAX (33), a functional analogue of vFLIP.

To test this hypothesis given the absence of a full-length structure of IKK γ (either in isolation or in complex with vFLIP and IKK α/β simultaneously), we used EPR spectroscopy to obtain distances between pairs and quartets of spin-labeled cysteines positioned at intervals along the length of the IKK γ molecule. The inter-label distance distributions observed for 56–95, 95–133, 133–169, and 158–186 toward the N terminus and 265–297 and 297–331 toward the C terminus are narrow, indicating a relatively rigid structure, while those observed for 186–218, 218–250, and 230–265 in the central region of the coiled-coil are broad indicating a more flexible structure. The distance distributions were subsequently used to generate a model of the entire IKK γ molecule as well as being analyzed for changes indicative of conformational rearrangements following incubation with either vFLIP or IKK β . Apart from stiffening

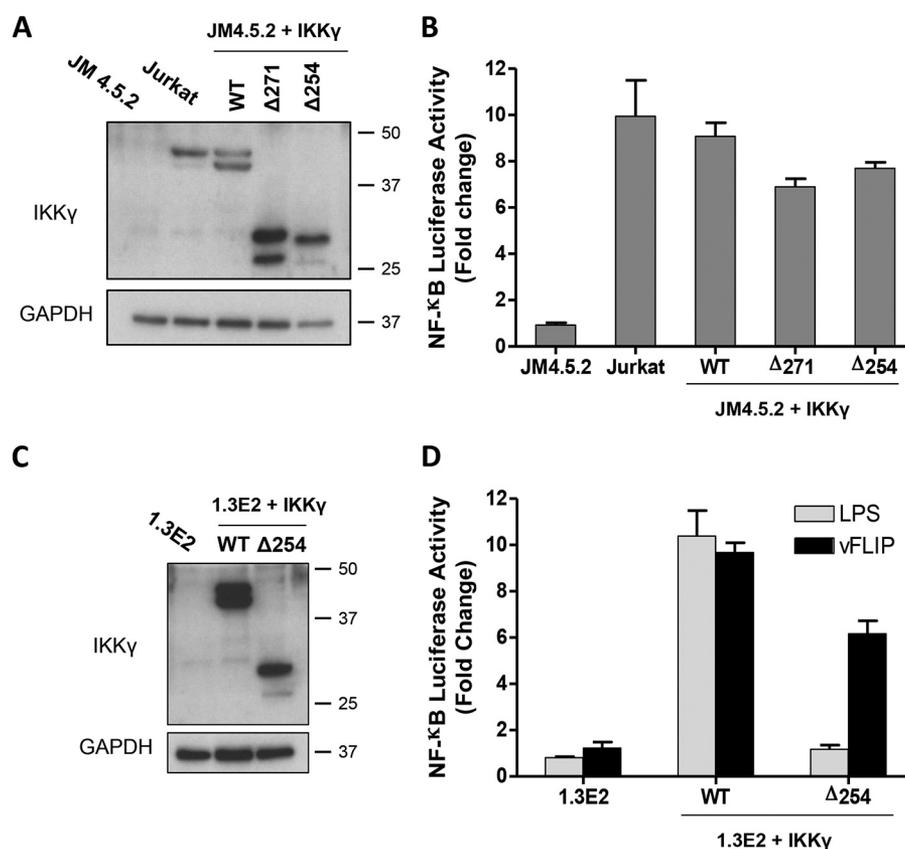


FIGURE 9. **NF- κ B luciferase reporter assays for the IKK γ $\Delta 254$ –419 and $\Delta 271$ –419 truncation mutants and D242R mutant.** *A*, Western blot analysis of IKK γ expression level in IKK γ knock-out human T-cells JM4.5.2, parental Jurkat cells and IKK γ wild type or mutant reconstituted JM4.5.2 cells. *B*, NF- κ B activation was measured using the BrightGlo Luciferase assay system 48 h after transduction with KSHV vFLIP LV (MOI = 50). Bars represent mean fold induction values \pm S.D. *C*, Western blot analysis of IKK γ expression levels in IKK γ knock-out mouse PreB cell line 1.3E2, parental cells 70Z/3, and 1.3E2 cells reconstituted with IKK γ wild type; $\Delta 254$ –419 mutant or IKK γ D242R. *D*, NF- κ B activation was measured using the BrightGlo Luciferase assay system, 6 h after stimulation with LPS (10 μ g ml $^{-1}$) or 48 h after transduction with KSHV vFLIP LV. Bars represent mean of relative luminescence unit (RLU) values \pm S.D.

of the coiled-coil observed in the vicinity of the vFLIP binding site, our results demonstrate that IKK γ does not undergo gross structural reorganization in response to binding. Furthermore, although the twisting observed for 133–169 (and the denaturation found for 95–133) indicates that subtle changes appear to be transmitted toward the N terminus of the molecule, no effect is observed for 56–96 in the vicinity of the IKK β binding site.

The main structural insight gained is that to accommodate the change in register between the N and C termini, the region between them contains a twist located around residues Lys-246 to Ser-248. This builds tension into the structure as neither region can find a low energy conformation. As observed by x-ray crystallography, this region is essential for formation of the IKK γ -vFLIP complex (12), which in turn leads to activation of the canonical NF- κ B pathway. The D242R mutation in this region appears to mimic the effect of vFLIP binding: formation of a salt-bridge to Glu-240 might induce a subtle change in structure that results in constitutive activation. The EPR data also demonstrate that binding of vFLIP stiffens this region of IKK γ .

Thus, it appears that although for the human cell, IKK γ is simply designed with different registers so that the N and C termini can perform their separate roles, namely kinase activity and regulation, respectively, the Kaposi sarcoma associated herpes virus has managed to exploit the structure of the transi-

tion between them in order to hijack the canonical NF- κ B pathway.

We note that bacterial chemoreceptors, another class of coiled-coil signaling proteins have been proposed to signal through subtle modifications of a frustrated domain (34). Given the importance of this motif in signaling proteins, it is intriguing to speculate that this might be a common mechanism within the wider superfold family.

Since the induction of conformational changes can now be excluded as a potential mechanism for vFLIP-mediated NF- κ B activation, alternatives need to be considered. Potentially, vFLIP may function by recruiting as yet unknown cofactors to IKK γ or through blocking those that are known to down-regulate the pathway in pro-inflammatory cytokine induced mechanisms, for example phosphatases (33). The former seems less likely considering recent reports where the absence of several factors essential to cytokine-induced activation failed to diminish vFLIP's capacity to activate the pathway (14). In agreement with this, truncation of IKK γ at Arg-254 in the region of altered helical register directly C-terminal to the vFLIP binding site has little impact on NF- κ B activation. Interestingly, a crystal structure of IKK γ has recently been reported in complex with Hoip, a component of the linear ubiquitin chain assembly ligase essential for IKK γ polyubiquitination (25). The Hoip-IKK γ interface encompasses residues in close proximity to this region

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which may therefore have an important role in NF- κ B activation by pro-inflammatory cytokines in contrast to vFLIP. Although the mechanism by which vFLIP activates the IKK complex remains unclear, given its apparent failure to induce anything but subtle conformational changes within IKK γ , we note that oligomerization of IKK β is key to autophosphorylation (35). This may suggest that vFLIP functions to promote oligomerization of the IKK β -IKK γ assemblies and that the D242R mutation mimics this action of vFLIP.

Author Contributions—C.B. performed cloning and site-directed mutagenesis. C.B., K.R., V.Z., and S.D. carried out expression and spin-labeling of proteins. C.B., K.R., V.Z., M.K., K.P., and C.K. performed the EPR spectroscopy. B.H. carried out the molecular modelling. M.B. performed *in vivo* assays. The manuscript was written by C.B., B.H., T.B., and C.K. with contributions from all authors. The study was conceived and supervised by M.C., T.B., and C.K.

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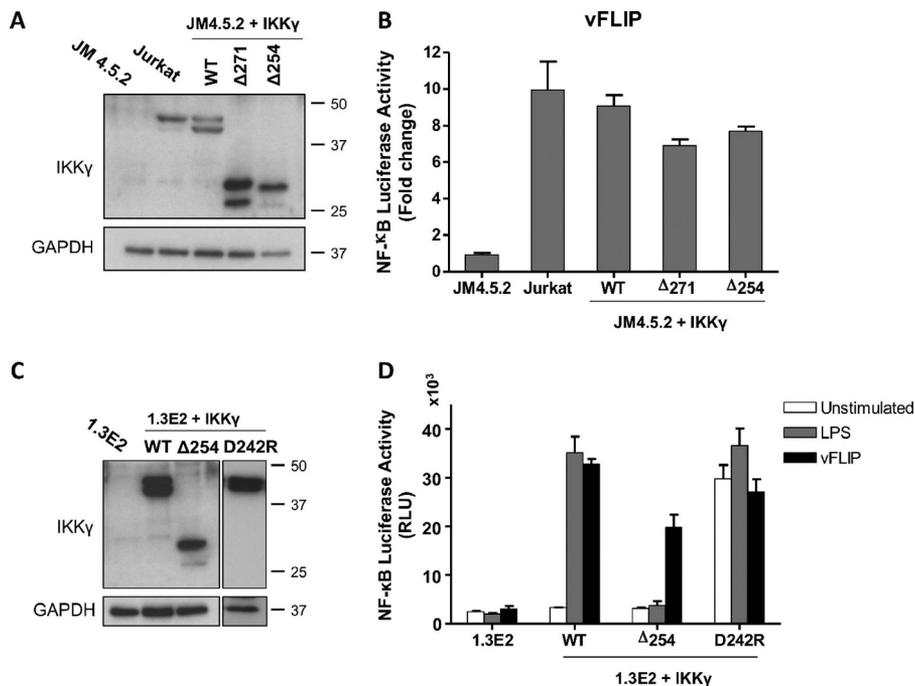
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PAGE 16547:

The panel showing IKKγ and GAPDH expression levels from 1.3E2 cells reconstituted with IKKγ D242R was omitted from Fig. 9C. The NF-κB activation data for the IKKγ D242R mutant was omitted from Fig. 9D. The complete figure is shown.



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