Inhibitor κB Kinase β Binding by Inhibitor κB Kinase γ†

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ABSTRACT: Activation of a large multisubunit protein kinase, called the inhibitor κB kinase (IKK) complex, is central to the induction of the family of transcription factors nuclear factor κB. IKK is comprised of two catalytic subunits, IKKα and IKKβ, and a regulatory IKKγ subunit. It is known that the catalytic IKKβ and regulatory IKKγ subunits associate through interactions mediated by the N-terminal region of IKKγ and an 11-mer peptide located near the C-terminus of IKKβ. In this study, we have defined the minimal IKKγ segment that binds IKKβ and determined the binding affinity of the IKKβ/IKKγ complex. We identified that the N-terminal segment spanning residues 40−130 of IKKγ binds the IKKβ C-terminal domain (residues 665−756) with $K_d \approx 25$ nM. Several smaller N-terminal IKKγ deletion mutants within the N-terminal 130 residues, although in some cases retained IKKβ binding activity, showed a tendency to aggregate and formed covalently linked complexes. However, expansion of the C-terminus of these fragments to residue 210 completely changed the solution behavior of the IKKγ N-terminus without affecting the IKKβ binding affinity. We also found that the IKKβ C-terminal domain formed a dimer in solution and the basic unit of the IKKβ/IKKγ complex was a dimer/dimer.

The NF-κB[1] pathway is a critical regulator of cellular response to a vast variety of stimuli including cytokines, growth factors, chemotherapeutic drugs, and bacterial and viral infections (*1*−*3*). In most resting cells NF-κB remains as an inhibited complex with inhibitor κB (IκB). Signal-dependent activation of a multisubunit protein kinase, known as the IκB kinase (IKK), specifically phosphorylates IκB in the IκB/NF-κB complex (*3*). Phosphorylated IκB becomes the target for polyubiquitination and subsequent degradation by the 26S proteasome. Free NF-κB regulates the expression of a variety of genes by binding to specific DNA sequences located in the promoters of a large number of target genes. The most striking feature of this signaling process is the rapid activation of NF-κB to coordinate the expression of most of the target genes. However, in most cases, these genes must be expressed only transiently to protect cells from autotoxicity resulting from unregulated growth, uncoupling the balance between apoptosis/proliferation and other deregulatory events. The IKK complex appears to be the key regulator of NF-κB activation (*4*). IKK represents a subclass of protein kinases that integrate a multitude of upstream signals and direct them to the activation of NF-κB.

The major cellular IKK complex is comprised of three subunits, two of which are the catalytic subunits, IKKα (also known as IKK1) and IKKβ (also known as IKK2), and the third of which, IKKγ/NEMO (NF-κB essential modulator), functions as an adaptor molecule (*5*−*8*). Hereafter, we will use IKKα, IKKβ, and IKKγ to refer to the three IKK subunits. The two catalytic subunits share extensive sequence homology and have identical domain organizations. A kinase domain is located at the N-terminal end followed by a leucine zipper (LZ) domain and a helix−loop−helix (HLH) domain (Figure 1a). The extreme C-terminal segments of IKKα and IKKβ are responsible for their association with the noncatalytic IKKγ subunit (NBD). The LZ domain is responsible for the dimerization of the catalytic subunits (*3*). When produced in eukaryotic expression systems, both these catalytic subunits can independently phosphorylate cognate IκB substrates. However, the activity of IKKβ, in particular, is mostly dependent on its association with the IKKγ subunit in vivo. Gene knockout experiments have clearly shown that both IKKβ and IKKγ subunits are essential for NF-κB activation by most proinflammatory stimuli (*4*, *9*, *10*).

The protein sequence-based secondary structure prediction method COIL (*11*) has identified distinct secondary structural elements in the IKKγ subunit. The N-terminal half of IKKγ is primarily a coiled coil (CC) where two long CC motifs are present. The first CC motif encompasses residues 50−196, which is followed by the second CC motif (residues 246−290) (*12*). The second CC motif is followed by an LZ motif (residues 302−350) and, finally, a zinc finger (Zn finger) motif at the C-terminus (388−419) (Figure 1A). In essence, most of the entire protein appears to be comprised of α helices. IKKγ functions as a bridging molecule between

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[1] Abbreviations: IKK, inhibitor κB kinase; NF-κB, nuclear factor κB; NEMO, NF-κB essential modulator; NBD, NEMO binding domain.

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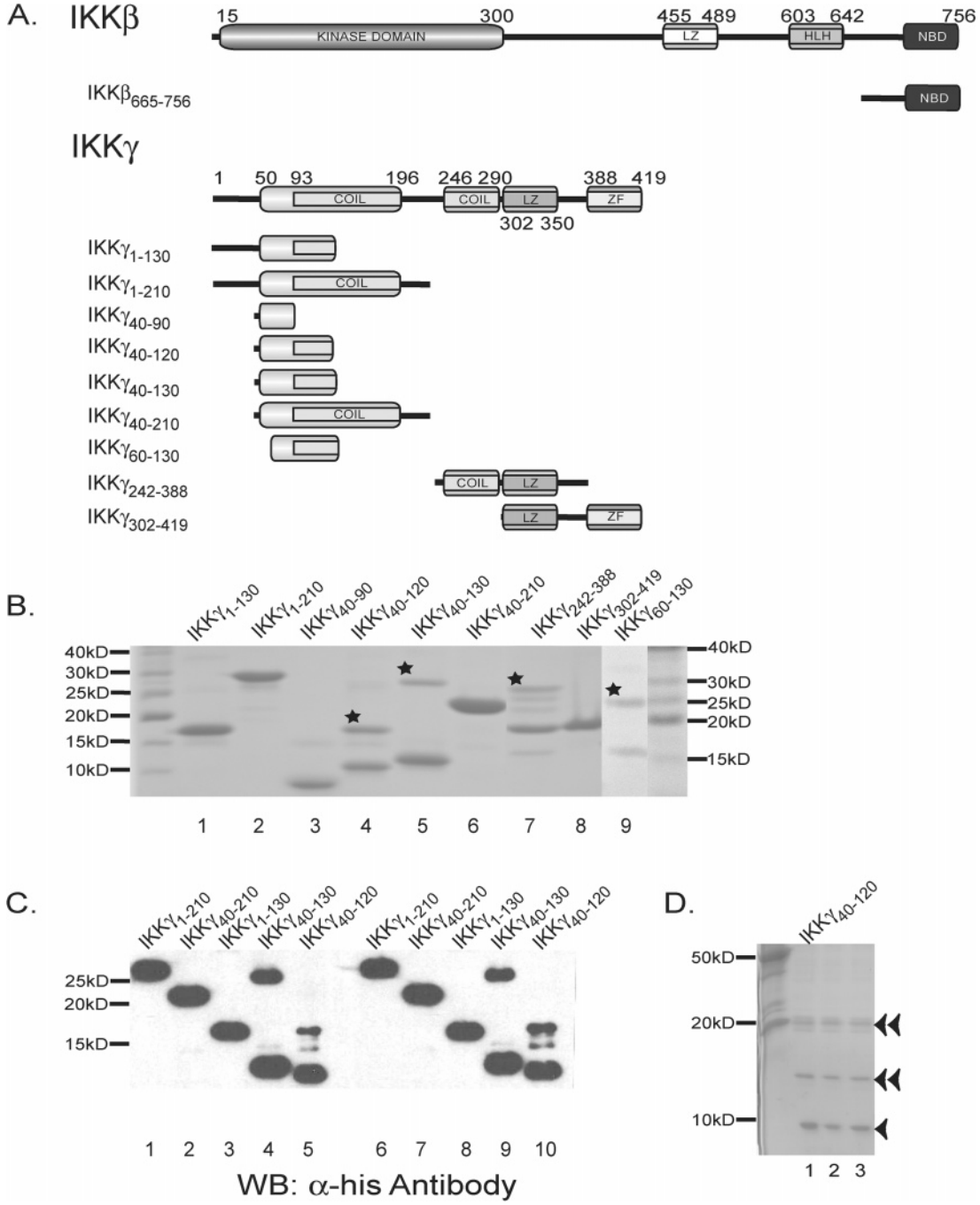

FIGURE 1: Domain organization, expression, and purification of IKKβ and IKKγ deletion mutants. (A) Domain organization of IKKβ and IKKγ and the deletion mutants of these IKK subunits. LZ, HLH, NBD, and ZF denote leucine zipper, helix−loop−helix, IKKγ binding domain, and zinc finger, respectively. (B) SDS−PAGE analyses of purified IKKγ fragments used in this study. The gel was stained by Coomassie Brilliant Blue. The asterisk denotes possible IKKγ-related high MW components. (C) Western blot analysis of the indicated IKKγ deletion mutants. (D) Coomassie-stained SDS−PAGE of IKKγ_{40-120} under denaturing conditions: lane 1, untreated; lane 2, 2× SDS; lane 3, 4 M urea.

the catalytic subunits and upstream kinases, allowing the activation of the catalytic subunits through phosphorylation of two serines in the activation loop (*13*). A second critical role of IKKγ that has been proposed is in the recruitment of the IKK complex to the membrane upon cytokine stimulation (*14*). The mechanism of IKK activation at the membrane is poorly understood. In vivo cross-linking, size exclusion chromatography, and sedimentation by analytical ultracentrifugation (AUC) experiments in vitro suggest that IKKγ can form a dimer, trimer, or tetramer (*15*−*18*).

The precise subunit composition of the most abundant 600−900 kDa IKKα/IKKβ/IKKγ complex is still unknown. There is clear evidence that other IKK complexes, such as the IKKα homooligomeric complex (*19*−*21*), the IKKβ/IKKγ complex (*22*), and perhaps the IKKβ homooligomeric complex, also exist in cells. These complexes modulate distinct signaling pathways to activate NF-κB (*4*). For example, IKKα is responsible for the activation of the NF-κB p52/RelB dimer in response to various stimuli. In addition, a recent report has shown that IKKγ also exists independent of the catalytic subunits and plays an important role in NF-κB activation by genotoxic stress (*23*, *24*). A short peptide spanning residues 734−744 of the IKKβ subunit was shown to displace IKKβ from the IKK complex, thus

inhibiting IKK activity and preventing NF-κB activation (*25*, *26*). A homologous peptide derived from IKKα was also able to dissociate the IKKα/IKKγ complex but with less potency than the IKKβ peptide in in vitro pull-down assays (*26*).

Although significant information has become available regarding the biological role of IKKγ in the NF-κB activation pathways, the biophysical properties of IKKγ and its affinity for the catalytic subunits are poorly studied. Considering that mutations within IKKγ are the only component in the NF-κB signal transduction pathways that are linked to human disease (*27*), it is important to understand how this molecule functions. It becomes necessary to examine in detail the interactions between the various subunits of the IKK complex. This study aims to use in vitro experiments with purified proteins to identify the IKKβ binding domain of IKKγ and to quantitatively examine the interaction properties between IKKγ and IKKβ.

EXPERIMENTAL PROCEDURES

Escherichia coli Expression Cloning of IKKγ/NEMO and IKKβ Mutants and Protein Purification. The PCR-amplified cDNA encoding different fragments of murine IKKγ was cloned into a T7 polymerase-based pET15b *E. coli* expression vector. Plasmids were prepared by the alkaline lysis method as described by Sambrook et al. Restriction analysis and DNA ligation were performed in accordance with the manufacturer's instructions. All truncations were confirmed by restriction mapping and DNA sequencing. *E. coli* DH5α was the recipient strain for the plasmids used in subcloning and sequencing. *E. coli* strain BL21(DE3) RP was used for the bacterial expression of pET constructs. Luria−Bertani (LB) medium containing 200 μg/mL ampicillin was used for growing *E. coli* cells.

Plasmids harboring the wild type and truncated constructs of NEMO/IKKγ were transformed into *E. coli* BL21 (DE3) competent cells. The cells were grown at 37 °C to an OD at A_{600} of 0.2−0.3 and induced with 0.1 mM IPTG for 12−16 h before harvesting. The cells were lysed by sonication in 20 mM Tris, pH 7.5, 0.5 M NaCl, 5 mM 2-mercaptoethanol (2-ME), 1 mM EDTA, protease inhibitor cocktail (Sigma), 0.1 mM PMSF, and 10% glycerol. The lysate was clarified by centrifugation and the protein purified by repeated binding to a Ni−NTA matrix, followed by a 40 mM imidazole wash and elution using 0.25 M imidazole. The eluates were further purified using size exclusion chromatography in 100 mM NaCl, 20 mM Tris−HCl, and 3 mM TCEP. Pure fractions were pooled and concentrated using a 3 or 10 kDa cutoff Centriprep (Millipore) and used for all further studies. IKK$\beta_{665-756}$ was prepared as both GST and His fusion proteins. To further test the nature of IKK fragments, a Western blot was performed using anti-histidine and anti-mouse as primary and secondary antibodies.

GST Pull-Down Experiments. Equal amounts of GST-tagged proteins and putative binding partners (10 μg each) were mixed in a buffered solution (20 mM Tris−HCl, pH 7.5, 0.5 M NaCl, 1 mM DTT, and 1% TritonX-100) and incubated for 40 min at room temperature before addition of a 15 μL slurry of glutathione Sepharose 4B (GE Health Sciences). The samples were then incubated for another 45 min at room temperature. The supernatant was removed after centrifugation followed by washing three times with dilution buffer. Samples were boiled with 1× SDS buffer, and bound proteins were separated by SDS−PAGE and visualized by Coomassie staining.

Isothermal Titration Calorimetry (ITC) Experiments. ITC experiments were carried out at 25 °C on a Microcal VP instrument. IKKγ and IKKβ proteins were prepared immediately prior to the experiment, by size exclusion chromatography on a Superdex S-200 30/10 column (GE Health Sciences) in 100 mM NaCl, 20 mM Tris−HCl, pH 7.5, and 3 mM TCEP (tris(2-carboxyethyl)phosphine hydrochloride) buffer. In a typical ITC experiment 20 or 15 μL injections of 50 μM IKKγ were made into a 5 μM solution of GST−IKK$\beta_{665-756}$ or His−IKK$\beta_{665-756}$ in the cell. Experiments were performed in duplicate or triplicate with different protein preparations. Heats of dilution of IKKγ into buffer were recorded in a separate experiment and subtracted from the binding data. Isotherms were analyzed using the Origin software (Microcal) as described elsewhere and fit to a model for a single set of binding sites.

Spectroscopic Studies. Far-UV circular dicroism (CD) measurements were made on an AVIV model 202 CD spectrometer. A two-piece Helma QS cuvette of path length 3 mm was used for all measurements. The spectra were recorded at 25 ± 2 °C in 0.1 M NaCl and 20 mM NaK_2PO_4 pH 7.5 buffer from 255 to 195 nm using a protein concentration corresponding to 9.1−0.5 μM for IKKγ_{1-210} and 9.5−0.5 μM for IKKγ_{40-210}.

Gel Filtration Analyses. Size exclusion chromatographic analyses were performed on a calibrated analytical gel filtration column (Superdex200 HR 10/30, GE Heath Sciences) using 100 μg of each protein in a 20 mM Tris, pH 7.5, 100 mM NaCl, 3 mM TCEP containing buffer to determine the oligomeric status of the purified proteins. Complexes between His−IKK$\beta_{665-765}$ and IKKγ_{1-210} or IKKγ_{1-130} were formed by mixing the two proteins with excess IKKβ. The protein mixtures were kept in ice for 1 h before being loaded (~200 μg of protein) onto the column.

Analytical Ultracentrifugation (AUC) Studies. Sedimentation experiments were carried by the Beckman XL-1 analytical ultracentrifuge. IKKγ_{1-210} (42 μM) was equilibrated for 16 h at 9000 rpm followed by 16 h at 17 000 rpm. Absorbance measurements were done by the absorbance optics of the centrifuge and were repeated following a 1 h time lapse to assess equilibration of the samples. Readings were taken at 280 nm. The data were analyzed using the Ultrascan software package.

RESULTS

Purification and Solution Behavior of IKKγ Deletion Mutants. Several published reports described the qualitative nature of the interaction between IKKγ and IKKβ. However, none of these studies used purified proteins in their in vitro experiments to examine the IKKβ/IKKγ interaction. To identify the region of IKKγ that retains the binding activity for IKKβ and to gain insight into their solution behavior in free states, we have generated several IKKγ truncation mutants. A schematic map of full-length IKKγ and different truncated IKKγ fragments used for this study is shown in Figure 1A. These fragments are expressed and purified from *E. coli* as soluble polyhistidine fusion proteins. We noticed

distinct solution behavior of these fragments during purification and after purification. We define the qualitative solution behavior as a function of protein precipitation during concentration in a buffer containing 0.1 M NaCl, 20 mM Tris−HCl, pH 7.5, and 2 mM DTT/10 mM 2-mercaptoethanol. Figure 1B demonstrates the purity of these proteins. IKKγ_{1-210} and IKKγ_{40-210} are two highly stable proteins that could be concentrated to over 20 mg/mL at different salt concentrations (50−500 mM NaCl) and pH values (6.5−8.5). The smaller IKKγ fragments displayed distinct solution behaviors; IKKγ_{1-130} and IKKγ_{40-130} showed temperature-dependent aggregation but behaved well. Further truncation of IKKγ_{40-130} at either end resulted in two fragments that were highly prone to aggregation. These smaller fragments also formed high molecular weight aggregates even under the denaturing conditions of SDS−PAGE (indicated by an asterisk) (Figure 1B). The ratio of the monomeric form to the high molecular weight protein was striking in the case of IKKγ_{40-120} or IKKγ_{60-130} (compare lanes 4−9 to the rest). The higher molecular weight forms were also clearly visible for the IKKγ_{40-130} and IKK$\gamma_{242-388}$ fragments. Western blot analysis showed that the high molecular weight bands were related to IKKγ (Figure 1C). The longer IKKγ deletion constructs including IKKγ_{1-130} did not reveal any high molecular weight band related to IKKγ. Prolonged boiling of the samples in the presence of a high concentration of DTT did not convert them into monomers (Figure 1C, lanes 6−10). To further examine the origin of these higher MW species, we treated IKKγ_{40-120}, which showed maximum amounts of high MW species, with a 2× higher concentration of SDS or with 4 M urea. The migration pattern of these treated samples remained unchanged as compared to that of the untreated sample (Figure 1D, compare lane 1 with lanes 2 and 3). A recent report has also described SDS-resistant oligomerization of the N-terminal domain of IKKγ (*28*). However, in that case the oligomer could be converted to a monomer after prolonged boiling, suggesting that oligomerizations were induced by disulfide bonds. It is possible that oligomers observed for the shorter fragments are also disulfide induced. However, unusual resistance to dissociation into monomeric species could be due to an unusual aggregation state of the truncated fragments.

The N-Terminal Region of IKKγ Interacts with the Catalytic IKKβ Subunit. It has been shown by in vitro GST pull-down and cell-based experiments that a small segment of only 11 residues (TALDWSWLQTE) near the C-terminus of IKKβ is sufficient to bind IKKγ (*25*, *26*). To test how the IKKγ fragments interact with IKKβ, we have expressed the C-terminal 91-residue (665−756) segment of IKKβ, which contains the 11-residue IKKγ binding peptide (NBP) region, as a GST fusion protein (GST−IKK$\beta_{665-756}$) in *E. coli* (Figure 1A). This fragment of IKKβ was generated on the basis of apparent proteolytic sensitivity near residue 665, which suggests that the C-terminal 91 residues may form an independent domain or substructure (data not shown).

We first broadly mapped the IKKβ binding region of IKKγ by performing GST pull-down experiments of GST-IKK$\beta_{665-756}$ with three fragments of IKKγ covering nearly the entire protein, IKKγ_{1-210}, IKK$\gamma_{242-388}$, and IKK$\gamma_{302-419}$. As expected, GST pull-down experiments demonstrated that only the N-terminal IKKγ_{1-210} fragment interacts with IKKβ (Figure 2A). To map the interaction region more precisely,

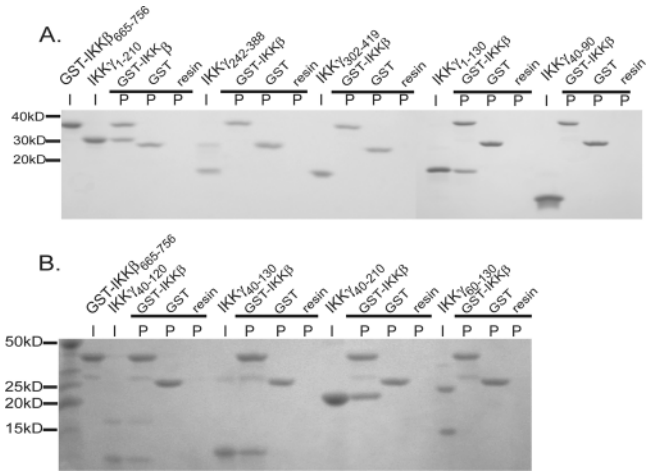

FIGURE 2: The N-terminus of IKKγ interacts with the C-terminus of IKKβ. (A, B) SDS−PAGE analysis of input and GST pull-down samples. Pull-down experiments using free GST or resin are the control experiments.

we have tested six other IKKγ truncation mutants we generated, IKKγ_{40-210}, IKKγ_{1-130}, IKKγ_{40-130}, IKKγ_{40-120}, IKKγ_{60-130}, and IKKγ_{40-90}. GST pull-down experiments revealed that IKKγ_{40-90} does not interact with IKKβ (Figure 2A). The first 40 residues from IKKγ_{1-130} (IKKγ_{40-130}) could be deleted without significantly affecting IKKβ binding. Although IKKγ_{40-120} retained at least partial binding activity for IKK$\beta_{665-756}$, IKKγ_{60-130} did not bind IKKβ with detectable affinity (Figure 2B). These results are mostly consistent with previous reports which demonstrated the N-terminal region encompassing the first 120 amino acids of IKKγ to be necessary for interaction with the catalytic IKKβ subunit (*14*).

Analysis of the Secondary Structure and Foldedness of the IKKγ N-Terminal Fragments. The secondary structure prediction method COIL (*11*) suggested that IKKγ exists primarily as a helical protein. CD was used to determine the nature of the secondary structure of the IKKγ_{1-210} and IKKγ_{40-210} fragments. Consistent with the prediction, far-UV CD spectra of all three fragments show double minima at wavelengths of 208 and 222 nm characteristic of an α-helical protein (Figure 3).

We carried out thermal melting analysis of these fragments to test the folding stabilities of the N-terminal fragments by monitoring the change in ellipticity at 222 nm as a function of temperature at pH 7.5. Helical properties were retained in both cases when the spectra were taken after completion of a cycle of thermal denaturation by heating and renaturation by cooling to room temperature. IKKγ_{1-210} and IKKγ_{40-210} showed a clear transition of helical content at ~40 °C. These experiments thus suggest that IKKγ_{1-210} and IKKγ_{40-210} are only marginally stable proteins with $T_m \approx 40$ °C. The folding transition is a reversible process indicated by the identical spectra of these fragments before denaturation and after renaturation. We have also carried out similar structural analysis with IKKγ_{1-130}, and it was shown that this fragment displays helical structure (data not shown). Unfortunately, melting analysis could not be done due to its sensitivity to aggregation (data not shown).

Oligomeric States of the IKKβ, IKKγ, and IKKβ/IKKγ Subcomplexes. We have further examined the oligomeric state of the two N-terminal IKKγ fragments IKKγ_{1-210} and

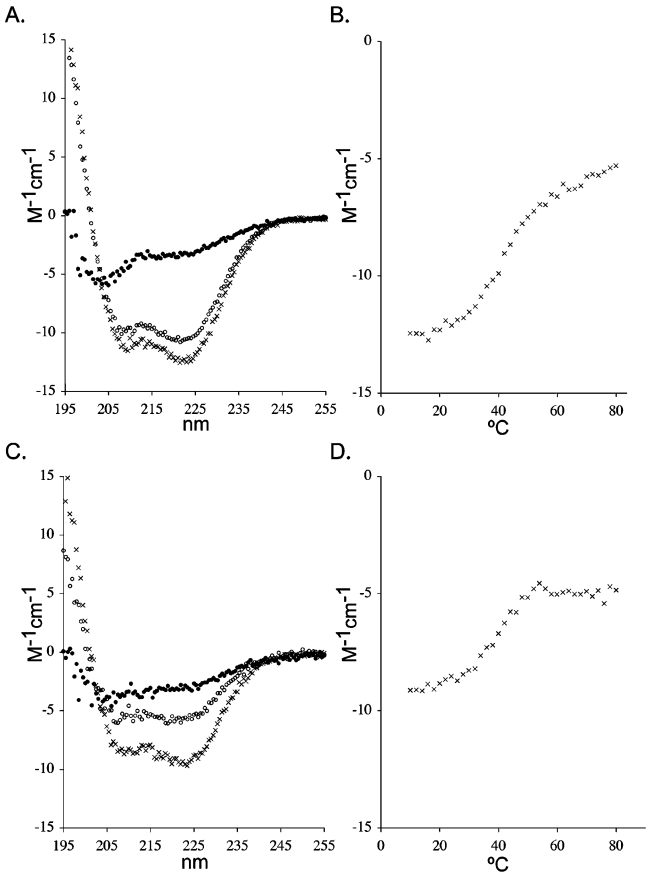

FIGURE 3: Secondary structure analysis and thermal stabilities of IKKγ fragments. (A) Far-UV CD spectra of IKKγ_{1-210} at room temperature (298 K). Spectra of the same sample at 90 °C. (B) CD spectra of IKKγ_{1-210} at increasing temperatures from 15 to 90 °C denoted by "×". Reversibility of melting analysis was done by slowly cooling the samples, and the spectra were measured (○). (C, D) Thermal stability of IKKγ_{40-210} monitored by CD as described for IKKγ_{1-210} in (A) and (B), respectively.

IKKγ_{1-130} by size exclusion chromatography on a Superdex 200 HR 10/30 analytical column. Because size exclusion chromatography is not a suitable method for the determination of the molecular weight of proteins of unknown shape, results from these experiments are interpreted cautiously. Both proteins eluted over a large volume, indicated by a broad peak (Figure 4A). This may indicate that each of these fragments exists in multiple oligomeric states, which are in equilibrium with each other.

We further wished to test the stoichiometry of IKKβ/IKKγ complexes. To this end, we purified the IKK$\beta_{665-756}$ fragment as a polyhistidine fusion protein from *E. coli* to avoid dimerization by GST. The size exclusion profile of His−IKK$\beta_{665-756}$ suggests that this fragment is a dimer in solution. Complexes between His−IKK$\beta_{665-756}$ and IKKγ_{1-210} or IKKγ_{1-130} were formed by mixing either of the two proteins with an excess of IKKβ. The mixture was then subjected to size exclusion chromatography. The elution profiles of these two complexes are shown in Figure 4A. The calculated molecular weights of IKKβ/IKKγ_{1-210} and IKKβ/IKKγ_{1-130} complexes are 235 900 and 114 300, respectively (Figure 4B). Considering IKKβ is a dimer, the 114 300 molecular weight species could be an octameric IKK$\beta_{665-756}$/IKKγ_{1-130} complex (calculated molecular weight of 118 840) where a tetramer of IKKγ possibly binds two IKK$\beta_{665-756}$ dimers. The IKK$\beta_{665-756}$/IKKγ_{1-210} complex could be a dodecameric complex where hexameric IKKγ_{1-210} binds three IKK$\beta_{665-756}$ dimers.

To further determine the oligomeric state of IKKγ_{1-210}, the protein was subjected to sedimentation experiments using analytical centrifugation under equilibrium conditions at 4 °C. The data set was fit globally by means of a nonlinear least-squares program to different models, ideal single species, monomer−dimer, monomer−trimer, monomer−tetramer, etc. However, none of the models fit the data very well for unknown reasons. Perhaps these coiled coil fragments do not behave well under conditions of sedimentation. The best model to which the data can be fit was the monomer−tetramer model for IKKγ_{1-130} and the monomer−hexamer model for IKKγ_{1-210} (Figure 4D,E). The oligomeric state of IKKγ_{1-210} observed here is consistent with the cross-linked dimer as reported recently (*28*). It is important to note that cell-based immunoprecipitation experiments could not differentiate between a dimer and an oligomer.

Binding Affinities of the IKKγ and IKKβ Complexes. We have carried out ITC experiments to measure the binding affinities of fragments of IKKγ to IKKβ (GST−IKK$\beta_{665-754}$) at 25 °C in 100 mM NaCl, 20 mM Tris−HCl, pH 7.5, and 3 mM TCEP. ITC is ideally suited to study this binding interaction because it measures the binding directly by monitoring the heat change associated with the binding interaction and requires no modification or labeling of the proteins. ITC is also considered one of the most accurate methods of detecting equilibrium dissociation constants in the millimolar to high nanomolar range. In addition, ITC provides a direct measure of the observed binding enthalpy (ΔH_{obsd}) and the stoichiometry of the binding interaction (n) (Table 2). Binding isotherms were obtained by titrating five different fragments of IKKγ (1−210, 40−210, 1−130, 40−130, and 40−90) with the GST fusion construct of IKK$\beta_{665-756}$ (Figure 5, Table 2). Binding was observed for all constructs except IKKγ_{40-90}. In all cases the binding stoichiometry was consistent with a 1:1 stoichiometry for all experiments. Given that the previous data showed the oligomeric state of the IKKβ fragment to be a dimer, this means that the interaction must be that of a dimeric IKKβ with a dimeric IKKγ. The data fit well to a model for a single set of binding sites, suggesting that there were no coupled equilibria between the binding of the dimeric IKKβ and tetrameric or hexameric IKKγ. The highest affinity interaction was for IKKγ_{40-210} and IKKγ_{40-130}, suggesting that the regions 130−210 and 1−40 are not essential for binding and in fact led to a reduction in binding affinity in these experiments. In all cases the binding was accompanied by a favorable ΔH_{obsd}. For IKKγ_{1-210}, the entropy change of binding (ΔS_{obsd}) was unfavorable, leading to a lower binding affinity. For IKKγ_{40-210}, ΔS_{obsd} was close to zero. For IKKγ_{40-130}, ΔS_{obsd} was favorable. The difference in the thermodynamic contributions to the overall free energy (ΔG_{obsd}) for the complexes appears to be compensatory, leading to a relatively small difference in the binding affinity of the different IKKγ constructs for IKKβ.

To further test whether the GST fusion tag had any effect in the binding interaction or stoichiometry of interaction, we repeated the ITC experiments using His−IKK$\beta_{665-756}$. In these experiments, we have used only two freshly purified IKKγ fragments, IKKγ_{1-210} and IKKγ_{40-210}. Similar to that

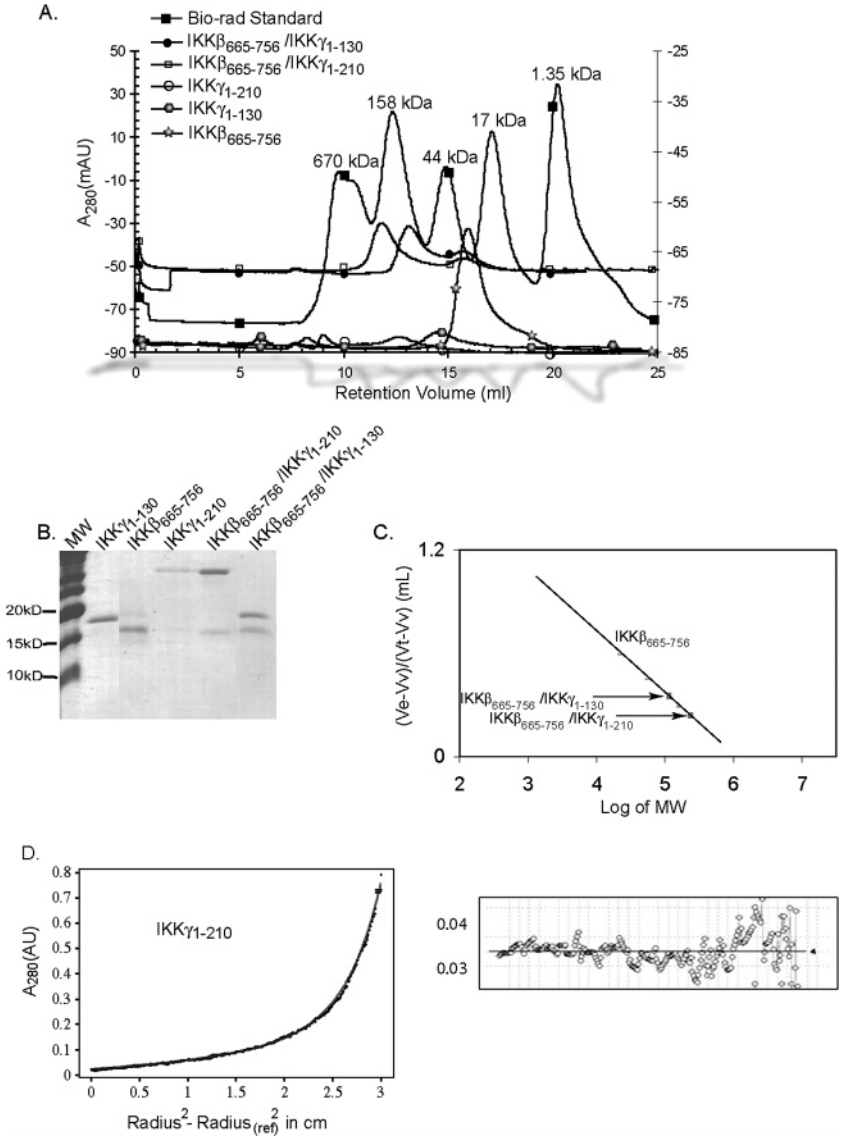

FIGURE 4: Determination of the molecular weights of unbound and IKKβ-bound IKKγ using size exclusion chromatography and analytical ultracentrifugation. (A) Chromatographic profiles of IKKγ, IKKβ, and the complexes between the IKKγ and IKKβ fragments. The BioRad standard contained a mixture of proteins with known molecular weights, thyroglobulin (670 000), aldolase (158 000), ovalbumin (44 000), and RNase A (17 000). The total column volume and the void volume of the column were determined by B12 and blue dextran (not shown here). (B) SDS−PAGE profiles of the peak fraction of each sample injected (stained by Coomassie). (C) MW analysis of free proteins and complexes from the standard curve. (D) AUC profiles of IKKγ_{1-210} (left) and residuals (right).

Table 1: Oligomeric States of Free IKK$\beta_{665-756}$, IKKγ_{1-130}, and IKKγ_{1-210} and Their Respective Complexes

	molecular weight	oligomeric state
IKK$\beta_{665-756}$/IKKγ_{1-130}	114 270	tetramer−tetramer
IKK$\beta_{665-756}$/IKKγ_{1-210}	235 900	hexamer−hexamer
IKKγ_{1-130}	55 980	not determined
IKKγ_{1-210}	159 000	not determined
IKK$\beta_{665-756}$	22 134	dimer

Table 2: ITC Data for Binding Affinities of Various IKKγ Fragments to IKKβ (GST− or His−IKK$\beta_{665-754}$)

IKKγ fragment	$K_{d,obsd}$ × 10^{-9} (M)	ΔH_{obsd} (kcal mol^{-1})	n	ΔG_{obsd} (kcal mol^{-1})	$T\Delta S_{obsd}$ (kcal mol^{-1})
1−210	110 ± 28	−14.7 ± 3.1	0.8 ± 0.01	−9.47	−5.23
40−210	16 ± 4.5	−11.2 ± 0.1	0.7 ± 0.01	−10.64	−0.56
1−130	83	−15.6	1.5	−9.65	−5.95
40−130	27 ± 5.1	−8.4 ± 0.9	0.9 ± 0.06	−10.32	1.92
40−90	NOB	NOB	NOB	NOB	NOB
1−210[a]	200 ± 18	−13.4 ± 1.6	1.3 ± 0.1	−9.1	−0.43
40−210[a]	69 ± 19	−10.1 ± 0.2	1.0 ± 0.03	−9.8	−0.33

[a] These fragments were tested against His-tagged IKKβ

of the GST fusion proteins, binding is driven by enthalpy with insignificant entropic contribution. We observed a 2−4-fold decrease in binding affinities as compared with those of GST−IKKβ (Figure 5 and Table 2). However, the difference is not significant compared to the variations in values we observed for different IKKγ proteins.

DISCUSSION

Although previous work identified the N-terminal region of IKKγ responsible for IKK interaction, the nature of this interaction remained elusive. In this paper, we describe the structural features of the IKKβ binding region of IKKγ and the interaction properties of the IKKβ/IKKγ complex. Secondary structure prediction methods indicated that the N-terminal region spanning residues 50−196 is α helical and a large portion of it can form a coiled coil structure. Consistent with this, we find that the N-terminal fragments

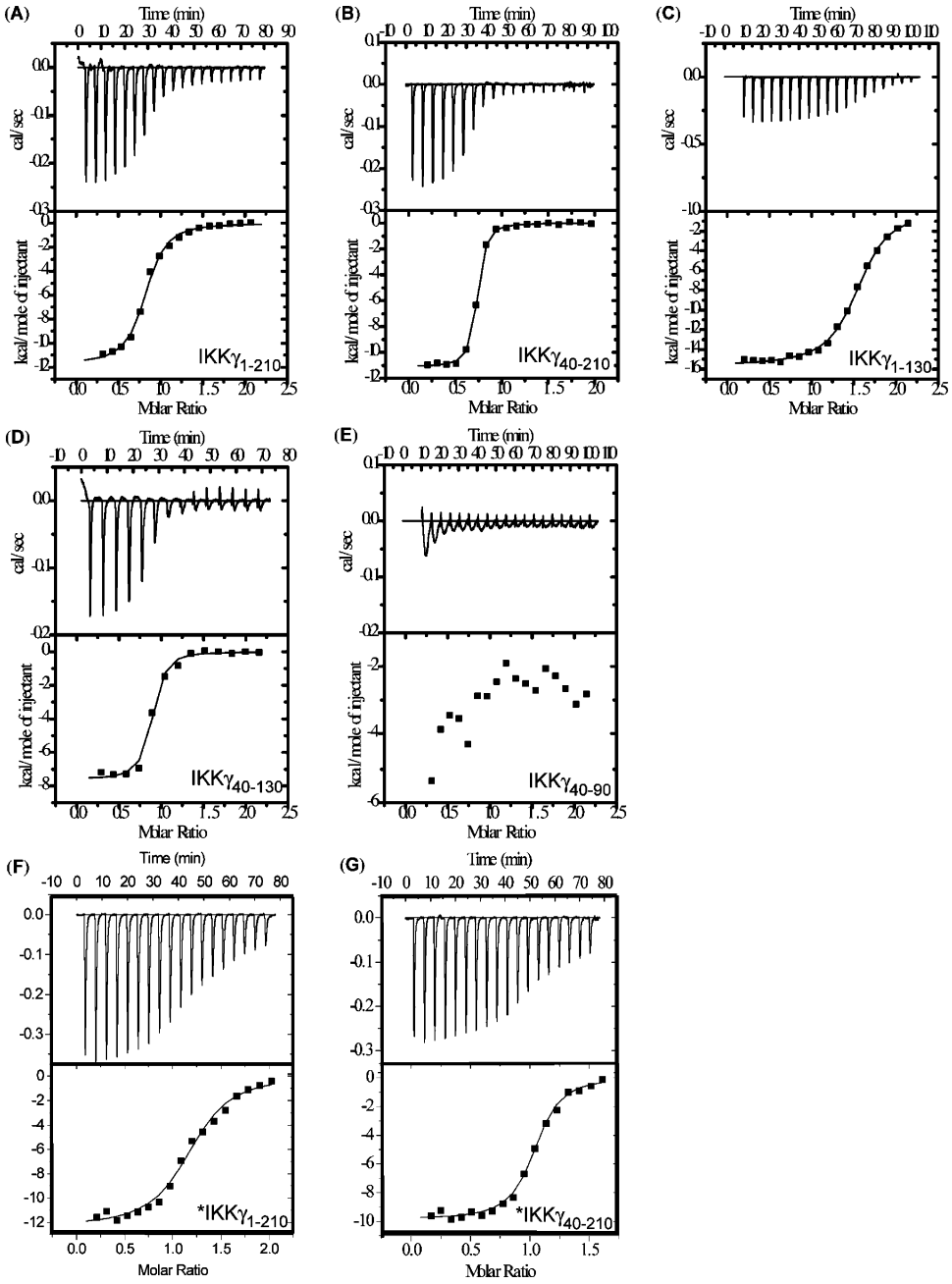

FIGURE 5: ITC binding isotherms for GST-IKK$\beta_{665-756}$/IKKγ complexes at 298 K: (A) IKKγ_{1-210}, (B) IKKγ_{40-210}, (C) IKKγ_{1-130}, (D) IKKγ_{40-130}, (E) IKKγ_{40-90}. ITC binding isotherms for His−IKK$\beta_{665-756}$/IKKγ complexes at 298 K: (F) IKKγ_{1-210}, (G) IKKγ_{40-210}. Data were analyzed using a 1:1 binding model (see the Experimental Procedures for details).

of IKKγ encompassing residues 1−210 and 40−210 (IKKγ_{1-210} and IKKγ_{40-210}) behave like a folded protein in solution. These fragments showed unfolding transitions around 40 °C. Although unfolding transition of well-folded globular proteins occurs at around 50−55 °C, significantly higher than that revealed by IKKγ_{1-210} and IKKγ_{40-210}, these IKKγ N-terminal fragments clearly contain a folded core. Further truncation at the C-terminus (IKKγ_{1-130} and IKKγ_{40-130}) rendered these proteins susceptible to temperature-sensitive aggregation. However, further deletion of IKKγ_{40-130} at the C-terminus (10 residues) or at the N-terminus (20 residues) resulted in smaller proteins that form cross-linked complexes, which suggests that IKKγ_{40-130} adopts a weakly folded conformation.

Sedimentation and size exclusion chromatography experiments demonstrate that both IKKγ_{1-130} and IKKγ_{1-210} are oligomeric proteins although their precise oligomeric states are different. We suggest that the assembly of IKKγ N-terminal fragments into higher oligomers is guided by differential and/or the weak folding stabilities of these fragments. Oligomerization is further enhanced and stabilized through covalent cross-linking when only the central part is retained in the construct (60−120). Surprisingly, the cross-linked oligomers of smaller fragments (IKKγ_{40-120} or IKKγ_{60-130}) were stable under SDS−PAGE in the presence of 5 mM DTT, high temperature, and 4 M urea. This suggests that these smaller fragments contain exposed residues that could undergo chemical reaction, most likely forming highly stable disulfide bonds. Consistent with this model, it has been shown recently that the region encompassing residues 50−70 might be primarily responsible for oligomerization and deletion of this region blocks NF-κB activation (*28*).

Recombinant full-length IKKγ apparently forms a large molecular weight complex on the basis of size exclusion chromatography experiments reported in the literature (*16*, *17*). Extensive studies have been done on the central part of IKKγ (IKK$\gamma_{242-388}$), which has been shown to exist as a trimer (*15*, *29*). It is possible to have two different regions with independent oligomeric propensities in isolation. Moreover, in the context of the full-length protein each of these regions can play a role in determining the overall oligomeric state of the protein. IKKγ has been shown to interact with a large number of signaling proteins in addition to the catalytic IKK subunits such as RIP, A20, TRAFs, TRADD, TAX, and CBP, to name a few (*30*, *31*). We cannot exclude the possibility that the physiological oligomeric state of IKKγ is determined by its interacting partners. Further experiments are required to understand the mechanism of IKKγ oligomerization.

We have determined the binding affinities of the complexes between IKK$\beta_{665-756}$ and the IKKγ N-terminal fragments. Although the mechanism is not clear, we observed that the presence of the first 40 residues is inhibitory to IKKβ binding. The equilibrium dissociation constants (K_d) of these complexes are approximately 25−100 nM. This reasonably stable association explains why IKKγ and IKKβ primarily coexist in resting cells. In the absence of any knowledge of cellular concentrations of these proteins, it is difficult to know the fraction of IKKγ that remains free in the cell. Unlike other extremely tight complexes such as IκBα/NF-κB and the p50/p65 heterodimer (low picomolar binding affinity), which ensures inactivation of all heterodimers, it is reasonable to assume that a fraction of IKKγ can freely exist in the cell. Indeed, IKKγ is known to exist free of IKKβ in vivo and participates in the signaling of genotoxic stress. In fact, the existence of a sumoylated or ubiquitinated form of IKKγ has been recently demonstrated in the literature and has been shown to significantly affect the NF-κB signal transduction pathway.

The stoichiometry of IKKβ/IKKγ complexes that emerged from our study is intriguing. IKK$\beta_{665-756}$ exists as a dimer in solution. Our chromatographic study demonstrates that a possible tetrameric IKKγ_{1-130} binds to two dimers of IKKβ. Hexameric IKKγ_{1-210} apparently binds to three dimers of IKKβ. Although these results appear to be inconsistent, a consistent 1:1 molar ratio of binding is observed. The fact that the C-terminal IKKγ binding domain of IKKβ is a dimer in solution led to the suggestion that the primary mode of binding is a dimer of IKKγ to a dimer of IKKβ (2:2). This conclusion is also consistent with a recent report which shows that the NTD of IKKγ forms a dimer and that dimerization is essential for its biological function (*28*). Further oligomerization of the basic tetrameric (dimer−dimer) complex might be artificial. Similar binding affinities of the octameric (4:4) IKKβ/IKKγ_{1-130} and dodecameric (6:6) IKKβ/IKKγ_{1-210} complexes suggest that the possible oligomerization of basic tetrameric (2:2) into $(2:2)_n$ complexes is noncooperative. Interestingly, modifications observed in the case of IKKγ_{40-130} do not affect its binding to IKKβ. We suggest that regions within the N-terminal domain of IKKγ provide IKKβ binding and that oligomerization and modifications are mostly independent events.

Both IKKβ and IKKγ contain separate oligomerization domains, the central CC/LZ domain (residues 246−350) in the case of IKKγ and the LZ/HLH domain in the case of IKKβ. These domains are expected to influence the oligomeric state of the native IKK complex. The calculated molecular weight of the tetramer is ∼300 000. This might be the basic unit of an IKK complex (dimer−dimer). This basic complex can undergo further self-assembly, forming an octamer (tetramer−tetramer). Such an oligomeric transition can be influenced by the CC/LZ domain of IKKγ and the LZ/HLH domain of IKKβ (Figure 6). Further higher order oligomer formation is also possible such as a dodecamer (hexamer−hexamer) or hexadecamer (octamer−octamer), varying the molecular weight from ∼300 000 for a tetramer to ∼600 000 for an octamer to ∼900 000 for a dodecamer. In induced cells, the molecular weight of the IKK complex increases from ∼600000−900000 to over 1 200 000 (*18*, *32*). In induced cells, IKKγ is modified by ubiquitin (Ub) (*33*). It is possible that octameric to dodecameric transition can be accomplished through Ub modification of IKKγ and recruitment of other factors.

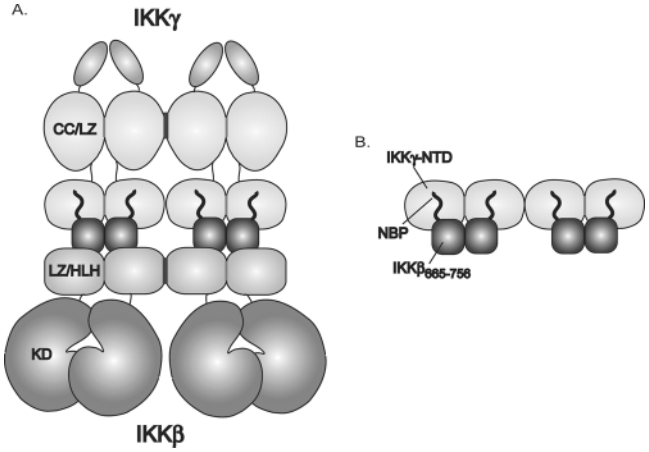

FIGURE 6: A model of oligomerization of the IKKβ/IKKγ complex. (A) A putative model of the IKKβ/IKKγ complex in an uninduced state. IKKβ and IKKγ are assembled in a 2:2 molar ratio. This assembly is stabilized by tetramerization of IKKβ and IKKγ. Further oligomerization of the complex can be mediated through other domains in induced cells. (B) The IKKγ NTD/IKKβ CTD tetramer is stable in isolation.

CONCLUSION

This paper is the first report of quantitative analyses of the binding interactions between catalytic and regulatory subunits of IKK using fragments of both subunits. Using qualitative studies, we also report how the octameric native IKKβ/IKKγ complex is assembled in resting cells which can then undergo further oligomerization upon stimulation of cells. This model can be used to further understand how upstream signals are transmitted through this complex and the importance of oligomerization in vivo. One of the key events that this study has not looked at is the lack of reduced binding interaction between IKKα and IKKγ. Considering the fact that the CTDs of IKKβ and IKKα are very similar in sequence, particularly in the IKKγ binding region, it is fascinating to know how IKKγ discriminates against IKKα. Future work is focused to understand this.

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