Mechanism of vaccinia viral protein B14 mediated inhibition of IκB kinase β activation

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Running title: B14 inhibits IKKβ activation

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Abstract

Activation of the IκB kinase β (IKKβ) is a central event in the NF-κB mediated canonical pro-inflammatory pathway. Numerous studies have reported that the oligomerization-mediated trans auto-phosphorylation of IKKβ is indispensable for its phosphorylation leading to its activation and the IKKβ-mediated phosphorylation of substrates such as the IκB proteins. Moreover, IKKβ’s interaction with NF-κB essential modifier (NEMO) is necessary for IKKβ activation. Interestingly, some viruses encode virulence factors that target the IKKβ to inhibit NF-κB-mediated antiviral immune responses. One of these factors is the vaccinia viral protein B14 that directly interacts with and inhibits IKKβ. Here, we mapped the interaction interface on the B14 and IKKβ proteins. We observed that B14 binds to the junction of the kinase domain (KD) and scaffold and dimerization domain (SDD) of IKKβ. Molecular docking analyses identified key interface residues in both IKKβ and B14, which were further confirmed by mutational studies to promote binding of the two proteins. During trans auto-phosphorylation of protein kinases in the IKK complex, the activation segments of neighboring kinases need to transiently interact with each other’s active sites and we found that the B14-IKKβ interaction sterically hinders the direct contact between the kinase domains of IKKβ in the IKK complex, containing IKKβ, IKKα and NEMO in human cells. We conclude that the binding of B14 to IKKβ prevents IKKβ trans auto-phosphorylation and activation, thereby inhibiting NF-κB signaling. Our study provides critical structural and mechanistic information for the design of potential therapeutics to target IKKβ activation for the management of inflammatory disorders.

Introduction

The inhibitor of κB kinase (IKK) complex plays a central role in regulating the activation of the nuclear factor (NF)-κB transcription factors, which are the master regulators of a variety of cellular processes, particularly immune and inflammatory responses(1). Although many different inducers activate NF-κB, most of the signaling pathways converge on the activation of the IKK(2). IKK is a large protein complex that comprises three major subunits, the kinase subunits IKKα (IKK1) and IKKβ (IKK2), and a regulatory subunit known as NF-κB essential modifier (NEMO, also called IKKγ). IKKβ harbors an N-terminal kinase domain (KD), an ubiquitin-like domain (ULD), a scaffold and dimerization domain (SDD), and a C-terminal NEMO-binding domain (NBD). It contributes to the majority of the IκB kinase activity of IKK and plays a dominant role in the canonical NF-κB pathway by phosphorylating IκBα. IKKα shares more than 50% sequence identity with IKKβ and plays an indispensable role in the non-canonical NF-κB pathway(3,4).

The main substrates of IKK are the IκB family of proteins, which are the inhibitory proteins of NF-κB (5). There are at least nine IκB members in the human genome, three of which (IκBα, IκBβ, and IκBε) are classical IκB proteins functioning as NF-κB inhibitors(6). The most extensively studied IκB member, IκBα, contains two serine residues in the destruction motif consensus DSGψXS (ψ stands for hydrophobic residue, X is any residue) in its N-terminal region. These two serine residues serve as the IKKβ phosphorylation sites. The doubly phosphorylated destruction motif is then recognized by the β-TrCP-SCF ubiquitin ligase for subsequent ubiquitination and degradation. After the degradation of IκBα the NF-κB is then released into the nucleus to regulate gene transcription(5,7).

Since its discovery in 1996, the function of IKK complex has been under intensive investigation. However its mechanism of activation remains elusive. In response to upstream signaling cues such as pro-inflammatory stimuli, the activation segments of IKKβ are phosphorylated at Ser177 and Ser181(7-9). In mouse embryonic fibroblasts (MEFs), some upstream kinases such as transforming growth factor β-activated kinase-1 (TAK1) have been shown to be required for IKKβ activation. However TAK1 only phosphorylates IKKβ at Ser177, which is a priming event that enables IKKβ to activate itself by phosphorylating Ser181(10). Other lines of evidences have pointed out that
oligomerization-mediated trans auto-activation is required for IKKβ activation. It has been found that the overexpressed and reconstituted IKK complex is active(11,12). And binding to different types of poly-ubiquitin chains via NEMO can activate the IKK(13,14). Indeed, in NEMO knockout cells, IKK activity is completely abolished(15). It is possible that activation of IKKβ requires its interaction with NEMO that in turn probably facilitates its oligomerization and trans auto-phosphorylation events(3,7). Our previous structural studies of IKKβ have indicated that the kinase domains in the IKKβ dimer face away from each other so that they are unable to trans phosphorylate each other(7). Therefore larger oligomeric forms must exist, so that the trans auto-phosphorylation event can take place.

NF-κB-dependent gene expression is essential for stimulating the pro-inflammatory and immune responses to defend viral infections, and viruses such as vaccinia virus (VACC) have accordingly evolved strategies to escape from the NF-κB-dependent anti-viral immune response by inhibiting the activation of IKK(16). VACV encodes a 17 kDa protein B14, which interacts directly with IKKβ to inhibit its activation and consequently blocking NF-κB activation(17). The viral protein blocks the phosphorylation of the activation segment of IKKβ to impede its activation but does not inhibit its kinase activity, as it has been shown to be incapable of inhibiting a constitutively active mutant form of IKKβ (S177E/S181E)(18). Interestingly, binding of B14 does not interfere with the assembly of the IKK holo-complex(19). In this study, we set out to elucidate the mechanism of VACV B14-mediated binding and inhibition of IKKβ activation. Study of this interaction has enlightened us on the mechanism of IKKβ activation from the perspective of a viral inhibitor. Dysregulation of the IKK/NF-κB pathway is associated with numerous diseases such as diabetes, cancer, inflammatory and autoimmune diseases(20). Therefore, our study has provided critical structural and mechanistic information for the future design of new therapeutics to specifically target IKKβ activation for the treatment of these devastating human diseases.

Results

B14 inhibits IKKβ activation but not its kinase activity

Previous studies have shown that B14 inhibits IKKβ phosphorylation on S177 and S181 of its activation segment(18). In this study, we used the HEK293T cell overexpression system to examine the human IKKβ auto-activation by assessing its phosphorylation on IκBα. In order to elucidate the role of B14-mediated inhibition of IKKβ activation, we co-transfected HEK293T cells with both Flag-IKKβ and HA-B14. IKKβ protein was then immunoprecipitated with anti-Flag antibody and its kinase activity was assessed in kinase assay with [γ-32P] ATP. The purified recombinant human IκBα protein was used as a substrate of IKKβ. Consistent with other studies(17,18), the viral protein B14 significantly inhibits IKKβ auto-activation in a dose-dependent manner (Fig. 1A). Since varying amount of B14 construct was co-transfected with the same amount of wild-type IKKβ construct, the inhibitory effect of B14 on IKKβ auto-activation can be manifested by its loss of activity on IκBα phosphorylation. In order to rule out the possibility that B14 also inhibits the activated IKKβ with dual phosphorylation on S177 and S181 in the phosphorylation of IκBα, we conducted an in vitro kinase assay with the purified recombinant IKKβ-SSEE (constitutively active S177E/S181E mutant). B14 does not show any distinguishable inhibitory effect on the IKKβ kinase activity (Fig.1B), confirming that it does not inhibit the active IKKβ.

B14 binds both the KD and SDD domains of IKKβ

In the structure of IKKβ, the KD, ULD and SDD domains interact with each other to form an integral tri-modular structural unit; however, the NBD has been shown to be an independently folded domain connected with a flexible linker to the rest of the protein(21). In order to map the B14 binding region on IKKβ, we used a glutathione S-transferase fusion B14 protein (GST-B14) to pull down the purified human IKKβ proteins (Fig. 2A). B14 binds to both full-length IKKβ and a construct without NBD (4-
675), indicating that NBD is not required for B14-IKKβ interaction. In addition, B14 binds to both the constitutively active IKKβ-SSEE and inactive IKKβ-D145N, showing that its activation state does not affect B14 association. Furthermore, S to E or S to A substitutions on S177 and S181 of the activation segment does not influence the B14 binding, suggesting that the binding may not engage the kinase activation segment.

To determine the B14 interacting domains on IKKβ, we expressed the GST fusion constructs of the KD (residues 1-307), KD-ULD (residues 1-384), ULD (residues 309-400), and ULD-SDD (residues 307-678) of human IKKβ in E. coli (Fig. 2B and D). The recombinant GST-SDD constructs were not soluble, indicating that when expressed alone separately the SDD domain cannot be folded properly. We used the GST-IKKβ fusion proteins to pull down untagged B14 protein. All constructs pulled down B14 except GST-ULD, which clearly demonstrates that the ULD is not required for the B14 binding to IKKβ. GST alone did not bind B14, indicating that the binding between B14 and other IKKβ constructs are specific (Fig. 2B). The NBD of IKKβ does not contribute to IKKβ binding to B14, because the GST-NBD (residues 675-756) IKKβ construct did not pull down B14 (Fig. 2C). Based on the results, we can conclude that B14 interacts with IKKβ on the junction between KD and SDD domains.

The M2 and M4 regions of IKKβ are required for its interaction with B14

Since the crystal structures of both the VACV B14 and IKKβ proteins are available, we conducted computational molecular docking to reconstruct the B14-IKKβ interaction with FRODOCK(22,23). In parallel we also used CLUSPRO(24,25) to search for the binding interface, where the surface of the KD and SDD junction of IKKβ was chosen as a space restraint. We have included both the xenopus IKKβ structure (PDB ID: 3QA8) in a closed conformation and a human IKKβ structure (PDB ID: 4E3C) in an open conformation for the modeling. We have identified the same potential binding surface composed of both KD and SDD of both orthologous IKKβ structures. Five surface regions of IKKβ, encompassing residues ranging from 179-197 (M1), 235-260 (M2), 408-416 (M4), 421-426 (M5), and 577-583 (M6), are contacting B14 in the docking models (Fig. 3A). To verify the docking models, we have substituted all the residues of M1 and M2 segments with Gly or Ser, mutated three key interacting residues F182K, V183K, and L186K of M3 on the GST-KD-ULD construct, and changed all the residues on M4, M5, M6 segments with Gly or Ser on the GST-ULD-SDD construct. The GST-fusion human IKKβ mutant constructs were then used to pull down the untagged B14 protein. We found that M2 and M4 mutants have almost 60% lower binding affinity when compared to the wild type constructs. M6 mutant has a light decrease in its interaction with the B14, while M1, M3, and M5 mutants have no significant changes in B14 binding (Fig. 3B and Fig. S1).

Based on the molecular docking modeling, the residues T31, Y35, L126, and F130 present on the surface of B14 can potentially contact IKKβ (Fig. 3C). F130 of B14 has previously been demonstrated to be required for IKKβ binding and inhibition(19), and in turn it validates our modeling results. In order to test which of the residues contributes to B14 interaction with IKKβ, we have created single substitution mutant constructs of GST-B14 to pull down the full length human IKKβ protein (Fig. 3D and Fig. S1C). Both the mutants T31K and Y35E demonstrate on significant decrease in binding affinity as the wild type B14 to IKKβ; L126E has a slight loss in binding, while F130K has negligible binding to IKKβ. This is consistent with a previous study showing that the B14 F130K mutant protein not only lost its binding to IKKβ but also was unable to inhibit NF-κB signaling(19). We postulate that F130 and L126 of B14 are likely to create a small hydrophobic surface patch to bind the KD and SDD junction surface of IKKβ in forming the inhibitory complex.

Binding of B14 to IKKβ does not block the kinase activation segment

We have used the identified interface to perform a local search with a molecular docking program ROSETTA(26,27). We have also included both
the *xenopus* IKKβ structure (PDB ID: 3QA8) in a closed conformation and a human IKKβ structure (PDB ID: 4E3C) in an open conformation for the modeling to indicate the evolutionarily conserved B14 binding. In these refined binding models, the small hydrophobic patch centered on F130 of B14 interacts with both M2 and M4 segments of IKKβ, which has no contact with the activation segment of the kinase domain (Fig. 4A-D). To verify this predicted binding interface, we created six full-length human IKKβ mutants with substitutions L259K, Y261A, V410K, L259K/Y261A, L259K/Y261A/V410K, and M2M4 mutant. These mutants were expressed in the human kidney embryonic 293 F (HEK293F) cells with an engineered N-terminal flag tag. The mutant recombinant proteins were used to pull down purified GST-fusion B14 protein expressed in *E. coli*. IKKβ L259K/Y261A/V410K mutant did not expressed in HEK293F cells, indicating that substituting all three residues disturbs its proper folding and stability. L259K and L259K/Y261A mutations severely weakened IKKβ binding to B14, while V410K or Y261A alone had minor effect on their interaction (Fig. 4E). Y261 is not conserved in *xenopus* IKKβ (Fig. 4C), indicating that Y261 is not critical. The M2M4 mutant has a similar binding pattern to B14 as L259K/Y261A. This shows that the three chosen residues in the M2M4 regions account for most of the binding affinity for B14. In this confirmed binding model of both the closed and open IKKβ structures, the activation segment of the kinase domain is exposed for substrate binding, which explains why B14 does not directly inhibit IKKβ kinase activity.

A model for B14 mediated inhibition of IKKβ activation

Our previous measurement of the recombinant full-length human NEMO and IKKβ proteins by size exclusion chromatography with multi-angle light scattering (SEC-MALS) has shown their dimeric status in solution. The measured molecular mass of the reconstituted IKK complex is around 2000 kDa, which corresponds to approximately 12-16 NEMO and IKKβ molecules in each complex assembly(28). Based on this study, we postulate that instead of one NEMO binding to one IKKβ molecule to form a heterodimer, NEMO uses each of its two kinase binding domains (KBDs) to interact with a NBD in two separate IKKβ molecules. In this mode of alternating binding, the NEMO protein crosslinks IKKβ to form a high-order oligomer, in which trans auto-phosphorylation and activation can occur (Fig. 5 and Fig. S2B).

Crystal structures of the IKKβ dimer have distinct closed and open conformations(7,29,30). There is a certain degree of flexibility in the SDD domain so that the KD and the proximal region of SDD can swing toward or away to adopt either a closed or an open dimer conformation (Fig. S2). In the *xenopus* IKKβ structure, although it is a S177E/S181E constitutively active mutant, the presence of a specific kinase inhibitor renders it in an inactive kinase form and IKKβ adopts a closed dimer conformation(7). Surprisingly, the active human IKKβ is in a unique open dimer conformation(29,30). IKKβ structure may vary between multiple transitional conformations in solution as previously proposed, or probably the closed conformation represents the inactive state while the open conformation indicates the active state.

We hypothesize that the IKKβ undergoes at least two transitional stages during its activation, a pre-activation complex and a post-activation (activated) complex. In the pre-activation complex, the activation segments of the neighboring KDs can contact each other for trans auto-phosphorylation (Fig. 5 and Fig. S2). It is possible that in the pre-activation IKK complex, NEMO “primes” IKKβ for activation by upstream signaling events, such as the poly-ubiquitin chain binding or phosphorylation by an upstream kinase(29,30). The dependence of the signaling for the IKKβ activation can be overcome by high IKK concentration, as NEMO/IKKβ complex can be activated when overexpressed(11). While in the post-activation complex, large conformational changes occur to allow the kinase domains to swing away from each other, making room for substrate binding and catalysis. Binding of the B14 to the junction of KD and SDD domains of the IKKβ causes a steric hindrance that impedes the optimal contact between KDs in the IKK complex. This structural rearrangement in turn blocks the insertion of the activation segment of one KD to
the active site of another for trans auto-phosphorylation (Fig. 5 and Fig. S3). B14 binds to both the constitutively active and inactive IKKβ proteins with comparable affinity (Fig. 2A). However, its binding to the pre-activation complex stops the KD interactions precluding trans auto-phosphorylation and activation. B14 can associate with the post-activation complex with little effect on the activity of the kinase (Fig. 1A). Differential binding and inhibition of IKKα and IKKβ by B14

B14 has been shown to have no observable phosphorylation. It also indicates that in the presence of the scaffold protein NEMO, the activation of IKKβ requires a well-defined structural architecture of the kinase complex that can allow optimal contact between the kinase domains.

**Impact of IKKβ oligomerization on its activation and B14 inhibition**

Although IKKβ stays as a dimer in solution, different types of oligomers have been observed in the crystal structures of both human and *xenopus* IKKβs(7,29,30). In the crystal structures of both *xenopus* and human IKKβs lacking the NBD domain, two IKKβ dimers interact on the KD proximal side of the structure to form a crystal packing tetramer. Since the activation segment of protein kinases has the potential to undergo conformational changes(31), the activation segment of one dimer is able to contact the active site of the other dimer for trans auto-phosphorylation in the observed crystal packing oligomers(7,29,30). In a crystal structure of human IKKβ without the NBD domain, the two KDs from two different IKKβ dimers form a V-shaped interface to permit trans auto-phosphorylation(29). Based on the modeling studies, with B14 binding to either IKKβ oligomers observed in the three different crystal structures, it blocks the contact of the KDs in all interfaces (Fig. S3). Under *in vivo* condition, IKKβ needs to be activated in the NEMO-IKKβ complex. But in the absence of an IKK holo-complex structure, it cannot be concluded that the observed KD interfaces in the crystal structures are relevant for IKKβ trans auto-phosphorylation *in vivo*. Our computational modeling of the B14-IKKβ interaction in all three crystal packing complexes indicates that the mode of B14 binding to IKKβ is an effective way to block the trans auto-phosphorylation between neighboring IKKβ dimers (Fig. S3). This is consistent with the experimental observation that co-expression of B14 and IKKβ inhibits the IKKβ trans auto-activation induced by overexpression (Fig. 1A).

**Differential binding and inhibition of IKKa and IKKβ by B14**

B14 has been shown to have no observable
binding to IKKα (18), which indicates that it specifically targets IKKβ to stop the anti-inflammatory canonical NF-κB signaling. Based on the sequence alignment of both human IKKα and IKKβ (Fig. S4A), the two important residue of IKKβ that are required for binding to B14, Y259 and V410, are conserved in IKKα. However, structural analysis of IKKα has revealed a huge structural variation on the corresponding B14 binding interface in IKKα (Fig. S4B), which explains the lack of B14 binding to IKKα. But the low resolution (4.5 Å) of the IKKα structure limits the reliability of this structural comparison to draw any conclusion (32). More structural, in vitro, in vivo binding studies, and kinase assays on B14 and IKKα interactions are entailed to address this question in the future.

Possibility of B14 inhibition of IKKβ activation by TAK1 phosphorylation

Some other studies have shown that the kinase TAK1 can activate IKKβ by directly phosphorylating the activation segment of IKKβ(30). It is not known whether the KD of TAK1 also forms a large multi-domain integral structure with the rest of the protein. Although it has been demonstrated that the KD alone of TAK1 is crystallizable and can be activated by binding to TAB, that is capable of forming an ensemble structure like IKKβ, but TAB is not necessary for the TAK1 kinase activity(33). A free KD domain of TAK1 therefore will have more rotation mobility and accessibility to contact the activation segment of IKKβ for phosphorylation than being held in a larger complex with other domains or proteins. In that case it is unlikely that the B14 binding will inhibit TAK1 phosphorylation of IKKβ. This implies that the phosphorylation by an upstream kinase is not the decisive step for IKKβ activation, and trans auto-phosphorylation is the main mechanism. Therefore, phosphorylation by TAK1 is merely a “priming” event, which may trigger or augment the IKKβ activation. It remains to be tested experimentally whether the presence of B14 indeed has any effect on TAK1 phosphorylation of IKKβ.

Future structural and in vivo studies to investigate the mechanism of B14-mediated inhibition of IKKβ activation

In this study, we have mainly focused on mapping the interaction interface between IKKβ and B14. For a deeper understanding of B14-mediated inhibition of IKKβ activation, structural characterization of both the NEMO-IKKβ and B14-NEMO-IKKβ protein complexes need to be carried out by x-ray crystallography or Cryo-electron microscopy. Structural comparisons between both the complex structures will provide more mechanistic and molecular insights about IKKβ activation and B14 inhibition. Furthermore, conducting in vivo studies to look for NF-κB signaling outcomes is necessary to improve our understanding of B14-mediated IKKβ inhibition.

Experimental procedures

Protein expression and purification

Various constructs of human IKKβ WT and the phosphor-mimic S177E/S181E mutants were designed with an N-terminal polyhistidine tag and a Tobacco Etch Virus (TEV) protease cutting site engineered between the tag and the protein. Recombinant IKKβ baculoviruses were made in DH10BAC cells, amplified, and were used to infect Hi5 insect cells in serum-free media (Invitrogen and Pharmingen). The cells were cultured in suspension and harvested after 48 hours of post-infection. The recombinant proteins were purified by Ni-affinity chromatography, anion-exchange and gel-filtration chromatography. The polyhistidine tag was cleaved by the TEV protease during protein purification.

All human IxBα and VACV B14 proteins were expressed in E. coli using pET28a and pGEX4T3 vectors and were purified by their respective affinity tags. GST-KD, GST-KD-ULD, GST-ULD and GST-ULD-SDD of human IKKβ were also expressed in E. coli using pGEX4T3 vectors. The tagged proteins were first purified with glutathione or Ni-NTA beads and their expression levels were assessed by SDS-PAGE.
Transfection, immunoprecipitation and kinase assay

The constructs Flag-IKKβ and mutants, HA-B14 were generated in the pcDNA3 vector using conventional PCR. HEK293T or HEK293F cells were transfected in with all the constructs using Lipofectamine 2000 (Invitrogen). After 24 hours, cell extracts were immunoprecipitated with anti-Flag antibodies bound to agarose beads (M2, Sigma). IKKβ kinase assays were essentially done as described (8,34). The immunoprecipitants were incubated with 2 μM full-length IκBα in 20 mM HEPES at pH 7.5, 10 mM MgCl₂, 20 mM β-glycerophosphate, 10 mM PNPP, 50 mM Na₃VO₄, 1 mM DTT, 20 mM ATP, and (1–10 mCi) [γ-32P]ATP at 30 °C for 30 minutes, and were subjected to SDS-PAGE and autoradiography. Immunoblotting was performed using anti-Flag (Sigma) or anti-HA (Sigma) antibodies (Upstate, 05-535). For in vitro IKKβ kinase assay, purified recombinant IKKβ-S177ES/182E mutant protein was mixed with varying amount of recombinant B14 or maltose binding protein (MBP) for kinass assay as describe above.

Pull-down assays

For GST pull down, the tagged proteins were purified with glutathione or Ni-NTA beads and followed by purification with gel filtration using a SuperdexG200 10/300 column (GE Healthcare). The expression levels were assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Beads containing estimated equivalent quantities of the GST-tagged proteins were mixed with the purified versions of the interacting partners. The mixtures were incubated at 4 °C for 2 hours with continuous rotation on a rocking platform. After centrifugation, the supernatants were removed. The beads were then washed three times with buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM β-mercaptoethanol, and 5 mM dithiothreitol), eluted with 25 mM Reduced Glutathione (GSH) and the elutions were subjected to SDS-PAGE analysis. All GST pull-down experiments were repeated at least three times for consistency.

For Flag pull-down, Flag-tagged human IKKβ and mutant proteins expressed in HEK293F cells were lysed in buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM β-mercaptoethanol, 5 mM dithiothreitol, 2% NP40 and cocktail protease inhibitor. The protein was then mix with anti-Flag antibody (Sigma), purified GST-B14 protein, and protein A/G Agarose (Thermo Scientific). The resultant protein complex was eluted by 200 μg/ml Flag-peptide (Sigma) and was subsequently used for Western blot with anti-Flag or anti-GST antibodies. The image was captured by G:Box Imaging Systems. All experiments were repeated at least three times.

Protein molecular docking

Two IKKβ structures (PDB code: 3QA8(7) and 4E3C(23)) were selected as the receptors and the B14 structure (PDB code: 2VVY(17)) was selected as the ligand. First, FRODOCK interactive protein-protein docking(22,23) (http://frodock.chaconlab.org/) online server was used for global search to provide the potential binding position information. Since GST pull-down experiments show that M2 and M4 segments of IKKβ are involved in binging to B14, and F130 of B14 is required for IKKβ interaction, we next used Cluspro 2.0 protein-protein docking(24,25) online server (https://cluspro.bu.edu/) to obtain more accurate complex models with these defined interfaces as restraints. The top ten models were analyses and the best fit one were chosen as an initial complex model for subsequent optimization with the Rosetta docking 2 (http://rosie.rosettacommons.org/) online server (26,27). Local docking was chosen as the docking protocol.

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Conflict of interest
The authors declare no conflict of interest. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author contributions
G.X. supervised the project; Q.T. and G.X. performed research; Q.T. and S.C. analyzed data; G.X., Q.T., and S.C. wrote the paper.

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Figure 1. VACV protein B14 inhibits IKKβ activation, but cannot impede its IκBα kinase activity. (A) In order to elucidate the role of B14-mediated inhibition of IKKβ activation, we analyzed the Kinase activity of auto-activated IKKβ in HEK293T cells co-transfected with full-length human IKKβ and varying amount of B14 construct. The empty vector pcDNA3 was used to adjust the total DNA of 55 µg used per transfection. (B) In order to rule out the possibility that B14 also inhibits the activated IKKβ with dual phosphorylation on S177 and S181 in the phosphorylation of IκBα, in vitro kinase assay with the constitutively active IKKβ-S177E/S181E (IKKβSE) and B14 proteins were conducted. Maltose binding protein (MBP) was used as a negative control. Each experiment was repeated three times.
Figure 2. Map the B14 interaction domains on human IKKβ. (A) Pull-down of IKKβ by GST-B14. GST protein alone was used as a negative control. IKKβ-SE1, IKKβ (1-756) S177E/S181E; IKKβ-SE2, IKKβ (1-678) S177E/S181E; IKKβ-DN, IKKβ (1-756) D145N; IKKβ-SA, IKKβ (1-756) S177A/S181A. (B, C) Pull-down of B14 by GST-IKKβ truncation mutant proteins. (D) Domain boundaries of the IKKβ constructs are shown in the diagrams, and the binding property between each IKKβ construct and B14 is summarized in the left column. Each experiment was repeated three times.
Figure 3. The M2 and M4 segments of IKKβ are required for B14 binding. (A) Molecular docking models of IKKβ-B14 interaction. The KD, ULD, and SDD domains of human IKKβ (PDB ID: 4E3C) are colored in yellow, pink and slate, respectively. The potential B14 interacting segments present on IKKβ protein are highlighted as M1-M2, M4-M6 and colored as cyan and blue, magenta, green and orange, respectively. Three residues, F182, V183, and L186 on M3 segment is shown in stick representation and...
colored in cyan. (B) Pull-down of B14 by GST-IKKβ mutants. GST protein alone was used as a negative control. GST-IKKβ-KD-ULD contains residues 1-384 of human IKKβ, and GST-IKKβ-ULD-SDD harbors residues 307-678 of human IKKβ, both of which are used as the wild type positive controls (WT1 and WT2). In addition, M1 mutant has residues 179-197 substituted with GGGSGGSS, M2 with residues 235-260 replaced with GGGSGGSS, M3 contains F182K/V183K/L186K mutations, M4 with residues 408-416 replaced with GGGGSS, M5 with residues 421-426 substituted with GGGGSS, and M6 with residues 577-583 replaced with GGGGSS. (C) The B14 structure is represented in ribbon diagram and colored in cyan, with the potential IKKβ interacting residues shown in stick representation and colored in magenta. (D) Pull-down of human IKKβ (1-675, S177E/S181E) by GST-B14 mutants. All experiments were repeated three times and yielded similar results.
**Figure 4.** B14-IKKβ binding interface. (A) and (B) Models of B14/IKKβ complex structures. Closed *xenopus* IKKβ (xIKKβ, PDB ID: 3QA8, (A)) and open human IKKβ (hIKKβ, PDB ID: 4E3C, (B)) structures were shown in ribbon and colored in green. VACV B14 was shown in ribbon and colored in cyan. M2, M4 and AS (Activation Segment of IKKβ) were colored in blue, magenta, and red, respectively. (C) and (D) B14-IKKβ binding interface for xIKKβ and hIKKβ, respectively. The key interacting residues are shown in stick. (E) GST-B14 was pulled down by human wild type (WT) and mutants IKKβ. The empty vector was expressed as a control. LY refers to the L259K and Y261A IKKβ double mutant. M2M4 IKKβ mutant contains both the M2 and M4 substitutions described in Fig. 3. The experiment was repeated three times with consistent results.

**Figure 5.** A model for B14-mediated inhibition of IKKβ activation. The KBD of NEMO interacts with the NBD of IKKβ that crosslinks IKKβ to a large oligomer. Upon activation by upstream signaling events or by high IKK concentration, the activation segments of the neighboring KDs can contact each other for trans auto-phosphorylation. Binding of the B14 to the junction of KD and SDD domains of the IKKβ causes a steric hindrance that impedes the optimal contact between KDs in the IKK complex, that in turn blocks the insertion of the activation segment of one KD to the active site of another for trans auto-phosphorylation and activation. KBD: kinase binding domain; NBD: NEMO binding domain; KD: kinase domain; ULD: ubiquitin like domain; SDD: scaffold and dimerization domain.
