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Understanding the Structural Basis of SIKE Interactions

Using Computational Approaches

by

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Honors Thesis

Submitted to

Department of biology

University of Richmond, VA

April 27, 2018

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Abstract

Tank Binding Kinase 1 (TBK1) acts as a catalytic hub in the regulation of numerous immune signaling pathways. Suppressor of IκB Kinase ε (SIKE) was recently characterized as a substrate of TBK1 whose binding properties are modulated by phosphorylation state, but very little is currently known about its function. However, because SIKE is known to form a dimer with itself, previous work generated a list of potential SIKE partners based on other proteins that contain sequences homologous to SIKE. Here, computational models of four potential SIKE partners, Heavy Chain myosin, tubulin, Beta Catenin and Ezrin, were generated and docked with SIKE in order to predict their possible mechanisms of interaction. These models revealed that theoretical interactions between SIKE and all four of these partners bear significant resemblance to the modeled interactions between SIKE and itself, supporting the conclusion that SIKE is likely to interact with these partners. Furthermore, analysis of the phosphorylation sites on SIKE demonstrated that they were all located very far from the interaction sites in three-dimensional space. Together, these results suggest that the three-dimensional conformation of SIKE is altered based on its phosphorylation state, and that SIKE may be activated by TBK1 signaling in order to regulate the cytoskeleton in response to immune challenges.

Introduction

The immune system exists in order to defend hosts against potentially dangerous pathogens. However, it must first be able to recognize that a certain pathogen is present before it can mount a response against that pathogen. In this way, depending on the type of pathogen present, different immune pathways may be activated. Specifically, in response to viral infections, the immune cells recognize some part of the virus, such as double-stranded viral RNA, and then secrete signal molecules in response to that viral recognition. Interferons (IFNs) are one such signal, and are vital to antiviral activity due to their ability to interfere with viral reproduction (Durbin 2000). Furthermore, IFN's additionally activate many other signals. For example, Type I IFN alters the cytokines released by immune cells. Type I IFN is also involved in anti-inflammatory pathways, where it mediates inflammation occurring in response to a viral infection so that the inflammation is not over-activated (Durbin 2000). Because it is so central to the immune response, interferon signaling is very tightly regulated by numerous signals and transcription factors (Huang 2005).

TANK-binding kinase 1 (TBK1), a non-canonical IκB kinase, is a critical convergence point in a wide variety of IFN signaling pathways, where it acts to upregulate the production of IFNs. While canonical IκB kinases phosphorylate IκB, allowing NF-κB to enter the nucleus and activate pro-inflammatory genes, TBK1 phosphorylates the interferon regulatory transcription factor IRF3 (Marion 2013). The TBK1 pathway is dependant on toll-like receptor 3 (TLR-3), which recognizes viral dsRNA released from lysed host cells. TRIF, which is associated with TLR-3, then interacts with TBK-1. Once activated by TRIF, TBK1 proceeds to phosphorylate IRF3. Phosphorylated IRF3 can then enter the nucleus of the cell, where it upregulates the transcription of IFN genes (Huang 2005).

In addition to mediating the Type I IFN response, TBK1 acts as a major catalytic hub which regulates a wide variety of immune responses. In 2005, Huang et al. identified a novel protein which they termed Suppressor of IκB Kinase ε (SIKE), that they determined acts as an inhibitor of TBK1. Later, Marion et al. (2013), investigated the mechanism by which SIKE interacts with TBK1. They found that SIKE binds to TBK1, forming a high affinity pseudo-dimer complex. In this state, TBK1 is less able to bind to and phosphorylate IRF3 due to the negative allosteric cooperativity resulting from SIKE binding. Furthermore, Marion et al. (2013) demonstrated that SIKE is actually a substrate of TBK1, not an inhibitor. SIKE contains six serine sites in a very similar arrangement to those in IRF3. Those six serines can be phosphorylated by TBK1, which alters the binding properties of SIKE to both TBK1 and, potentially, other proteins (Bell in press).

The fact that SIKE is a substrate of TBK1, rather than simply a competitive inhibitor, led Marion et al. (2013) to hypothesize that phosphorylated SIKE could potentially act to organize other immune processes. Because they found that SIKE localized to actin cytoskeletal structures, and that SIKE has high sequence homology with the actin cytoskeleton-associated protein IRF3, they believed it was likely that SIKE is involved in organizing immune cell cytoskeletons in response to pathogens. Furthermore, as shown by Marion et al. (2013), SIKE interactions with TBK1 were regulated by the phosphorylation of SIKE at six serine residues, supporting the hypothesis that pSIKE interacts with other proteins differently from SIKE with no post-translational modifications. This hypothesis was later confirmed by Bell, JK (in press), where it was shown that an interaction between SIKE and tubulin was modulated by the phosphorylation state of SIKE.

The cytoskeleton is important in the immune response, as well as many other cellular functions, because it controls cell movement and intracellular transport. Some of the most important cytoskeletal proteins include heavy chain (HC) myosin, Ezrin, β-catenin, and tubulin. Tubulin itself is one of the major constituents of the cytoskeleton, as tubulin filaments polymerize to form microtubules. These microtubules provide an intracellular network on which motor proteins can travel in order to transport molecules to different areas of the cell (Farache et al. 2018). Myosin heavy chain binds to actin filaments and contains an ATPase domain. When it hydrolyzes ATP, the myosin head pulls on actin, resulting in contraction. This contractile mechanism generates the force necessary for processes such as motility and cell adhesion (Dulyaninova and Bresnick 2013), both of which are necessary for immune cell function.

Ezrin and β-catenin are both involved in cell adhesion through the cytoskeleton. Ezrin, a member of the ERM protein family, is a membrane protein that binds to the actin cytoskeleton. In this way, Ezrin is itself a vital regulatory protein that links cytoskeletal processes to membrane processes. This bridge allows the cytoskeleton to change in response to external signals. Specifically, it is involved in cell movement and adhesion, among others, and has even been implicated in the metastatic movement of cancer cells (Clucas and Valderrama 2014). Similarly, β-catenin is involved in epithelial-mesenchymal transitions. β-catenin is activated through Wnt signaling and binds to the intracellular domain of cadherin. Once bound to cadherin, β-catenin also binds to α-catenin, which associated directly with the actin cytoskeleton. Because it is important in cell adhesion and epithelial-mesenchymal transitions, misregulation of β-catenin has also been linked to cancer cell metastasis (Bienz 2005).

Recently, Bell, E (unpublished observations) investigated the potential for SIKE to interact with other proteins. By generating a list of proteins with significant amounts of

sequence homology to SIKE, it was possible to predict which proteins may bind to SIKE, since proteins are most likely to interact with other proteins in areas with homologous residue structures. This list included proteins such as HC myosin, Ezrin, β-catenin, and tubulin. Tubulin and alpha Actinin have experimentally been shown to interact with SIKE (Bell, JK. in press). These potential partner proteins were subdivided into groups of those that have predicted coiled-coil regions, such as HC myosin and Ezrin, and those that do not, such as β-catenin, and tubulin. SIKE itself is predicted to contain a large coiled-coil, which is commonly a binding site for protein-protein interactions. Since each of these four proteins is vital to maintenance of the cytoskeleton, these findings further support the concluding hypothesis of Marion et al. (2013) that SIKE acts on the cytoskeleton. While the mechanisms through which SIKE could affect these proteins is not yet understood, it is possible that SIKE acts as a link between the TBK1-mediated immune response and the cytoskeleton.

Here, we examine the potential SIKE interaction partners HC myosin, Ezrin, β-catenin, and tubulin, and whether their interactions with SIKE are modulated by either direct or indirect interaction with phosphorylated SIKE residues. We demonstrate that it is very likely that these proteins interact with SIKE and characterize the interacting residues involved with each partner. Furthermore, the results suggest that phosphorylation of SIKE most likely results in a three-dimensional conformational change in SIKE, and that this different conformation alters how SIKE interacts with its partners.

Methods

In order to model the interactions of SIKE with its potential partners, it was necessary to first model the structures of those potential partners that had not yet been crystallized. The predicted model from the sequence of SIKE itself was provided by Bell, JK based on her previous work (in press). Several sequences for each partner, each coming from a different species or a different isoform, were taken from the Protein Database (Berman et al.) if available (Kim et al.), or the PubMed protein section if not. Multiple sequences for each protein were used in order to ensure consistency of later results, since minor polymorphisms are often present in homologous proteins between different species.

The many sequences were compared to SIKE through clustal analysis generated by the Clustal Omega server (Sievers et al.). The clustal results were analyzed in order to determine which regions of the protein contained the highest amount of sequence homology to SIKE.

Sequences of sections of the partners that were highly homogenous with SIKE were then modelled using the iTasser web service (Yang; Roy et al.; Yang et al.). Due to constraints with the maximum sequence length the server could handle, it was necessary to identify a relatively small region that was most likely to bind to SIKE.

Structure accuracy was confirmed through Ramachandran plot analysis generated by RAMPAGE (Lovell et al. 2003). The most likely structures generated by iTasser were compared to those listed as less likely. By comparing the number of residues in the allowed and forbidden regions between different models, it was determined that the top models were the most likely to be valid.

Once the homologous partner regions had been modeled, they were docked with SIKE using the ClusPro web service (Kozakov et al; Comeau et al.). The docked models were then examined in order to determine which residues on both the partner and SIKE were interacting. Multiple potential models for each partner were docked with SIKE, and the binding areas between models were compared to ensure that they were consistent between the most likely models.

Docked SIKE models were visually analyzed with PyMOL (Schrödinger LLC) in order to determine the individual residues that were interacting. The interacting residues in both SIKE and its partner were recorded and compared between partners to determine whether certain residues appeared frequently. Finally, potential changes in interaction depending on SIKE phosphorylation state were predicted based on the proximity of interacting residues to SIKE serine residues.

Results

SIKE is known to form a dimer with itself in vivo through interactions within its coiled-coil region. Therefore, it is likely that other proteins containing high homology to SIKE could also act as partners to form a dimer with SIKE. Previous work by Bell (in press) demonstrated that tubulin, Beta Catenin, Ezrin and HC myosin all contain high sequence homology with SIKE, and are therefore potential partners to dimerize with SIKE. Due to constraints in available protein modeling services, however, it was not possible to model the entirety of these proteins. Therefore, clustal analysis was performed in order to isolate the sections of each potential partner with the highest homology to SIKE (Figure 1). These sections would, theoretically, be the most likely SIKE binding sites.

Consistent with the work of Bell, E (in press), clustal analysis of all four potential partners revealed areas with significant SIKE homology. The sequences of these areas were then used to create models that would later be docked with SIKE. Additionally, the modeled sections for Ezrin and HC myosin both contained coiled-coil regions. These predicted coiled-coil regions are significant because, while coiled-coil regions themselves are often sites of protein interactions, SIKE is known to interact with itself in a coiled-coil region. The fact that the sections of these proteins that contained high SIKE homology also contained coiled-coil regions lends further credence to the hypothesis that these sequences are where SIKE is likely to interact on the respective partners. The tubulin and Beta Catenin models did not contain coiled-coil regions, as was expected.

In order to verify the validity of the models, Ramachandran plot analysis (Figure 2) was performed on each of the top five most likely models output by iTasser. Phi-psi analysis confirmed that the top model output for each partner sequence contained more residues in the

favored region and fewer in allowed and outlier than less likely outputs. Therefore, it was concluded that the top models, which were used for the subsequent steps, were in fact the most likely to exist in reality.

Each of the four potential partners were then docked with SIKE in order to model how they might interact (Figure 3). In addition, SIKE was docked with itself in order to serve as a positive control, and Malate Dehydrogenase, which was not theorized to interact with SIKE, was used as a negative control. The SIKE-SIKE model showed interactions along the coiled-coil regions of both SIKE proteins, which was consistent with previous observations and served to validate the docking procedure.

In both the Ezrin-SIKE and HC myosin-Sike models, interactions occurred within the coiled-coil regions of both the partner and SIKE. However, the phosphorylatable serine residues on SIKE, starting with S-135, were all very far away from the site of interaction in three-dimensional space in both models. If the phosphorylatable serines were present in the interaction site itself, this would suggest that residues on the partners could interact differently with phospho-serine, and that this different interaction could alter the properties of dimer formation. However, the fact that the phosphorylatable serine residues were all very far away from the interaction site suggests that phosphorylation of SIKE must result in a larger, three-dimensional conformation change in SIKE, and that this different SIKE comformation interacts differently with its partners than non-phosphorylated SIKE (Figure 4).

Similarly, although tubulin and Beta Catenin do not contain coiled-coil regions themselves, they interacted with SIKE in its coiled-coil region. Furthermore, the phosphorylatable SIKE residues were also located far from the interaction site in three-dimensional space. This once again suggests that a three-dimensional conformational change in

SIKE structure is necessary in order for phosphorylation to alter SIKE binding properties. Finally, it demonstrates that the mechanism of SIKE modular interactions may be independent of the presence of a coiled-coil region in the partner protein.

The interactions present in the SIKE-SIKE dimer model were used as a baseline in order to determine which residues would likely be important in interactions between SIKE and proteins with homologous sequences. Visual analysis of the interaction sites in the SIKE-partner models revealed that several residues frequently involved in SIKE-SIKE binding were also involved in interactions between SIKE and other partners (Figure 4). These results further support the hypothesis that these four partners are likely to interact with SIKE in the way that they were modeled here. Additionally, this analysis discovered a pattern of several residues of interest that are likely involved in SIKE interactions. Interestingly, Malate Dehydrogenase also interacted with SIKE on many of the same residues, although several are antagonistic in its case.

Discussion

These results demonstrate that it is likely that SIKE interacts with Beta Catenin, HC myosin, Ezrin and tubulin. Many of the residues present in the SIKE-SIKE interaction were also present in interactions between SIKE and its partners. Because it is already known that SIKE forms a dimer with itself, this suggests that these partners are likely to actually interact with SIKE. This hypothesis is further supported by the fact that all of these partners contain high sequence homology to SIKE to begin with. However, although previous work has demonstrated that the phosphorylation of SIKE alters its affinity for tubulin (Bell in press) and TBK1 (Marion et al. 2013), this study does not demonstrate any evidence that phosphorylation state has a direct effect on SIKE interactions with any of the partners explored herein, including tubulin. Rather, it seems likely that phosphorylation of SIKE leads to a three-dimensional conformational change of the protein, and that this altered conformation interacts differently with partners than non-phosphorylated SIKE.

Finally, this study demonstrates that SIKE is likely to function in regulation of the cytoskeleton in response to immune system challenges. All of the potential SIKE partners tested here are involved in cytoskeleton processes. Furthermore, SIKE is phosphorylated by TBK1. TBK1 is a vital catalytic hub in the innate immune response that is activated when toll-like receptors on an immune cell bind to viral dsRNA and acts on a large number of other proteins. As such, the central role of TBK1 is to coordinate the activation or deactivation of a large number of other pathways in response to the presence of a pathogen. While the most well-characterized function of TBK1 is to activate interferon regulatory factors, which then proceed to upregulate interferon genes, there is a lot more than this that goes into an immune response. Combined with the fact that SIKE localizes with the actin cytoskeleton (Marion et al. 2013), the

fact that many potential partners of SIKE are involved in cytoskeletal regulation suggests that SIKE is most likely involved in controlling the cytoskeleton in order to carry out an effective immune response.

While the computational results shown here suggest that these four proteins are likely to bind to SIKE, only tubulin has yet to be tested experimentally. Future work on SIKE should focus on validating the results herein through in vitro assays that will determine whether or not these proteins truly do bind to SIKE. In addition, the assay that formed the basis for this work only investigated proteins with sequence homology to SIKE. While this homology does suggest that these are potential partners for SIKE, it does not generate an exhaustive list. It is quite possible that other, as of yet unknown SIKE partners exist that contain very little homology to SIKE itself. Furthermore, it is not currently known by what mechanism phosphorylation of SIKE alters the binding properties of SIKE. While these results suggest that phospho-SIKE undergoes a conformational change, it is not currently known what this change might entail.

Figures

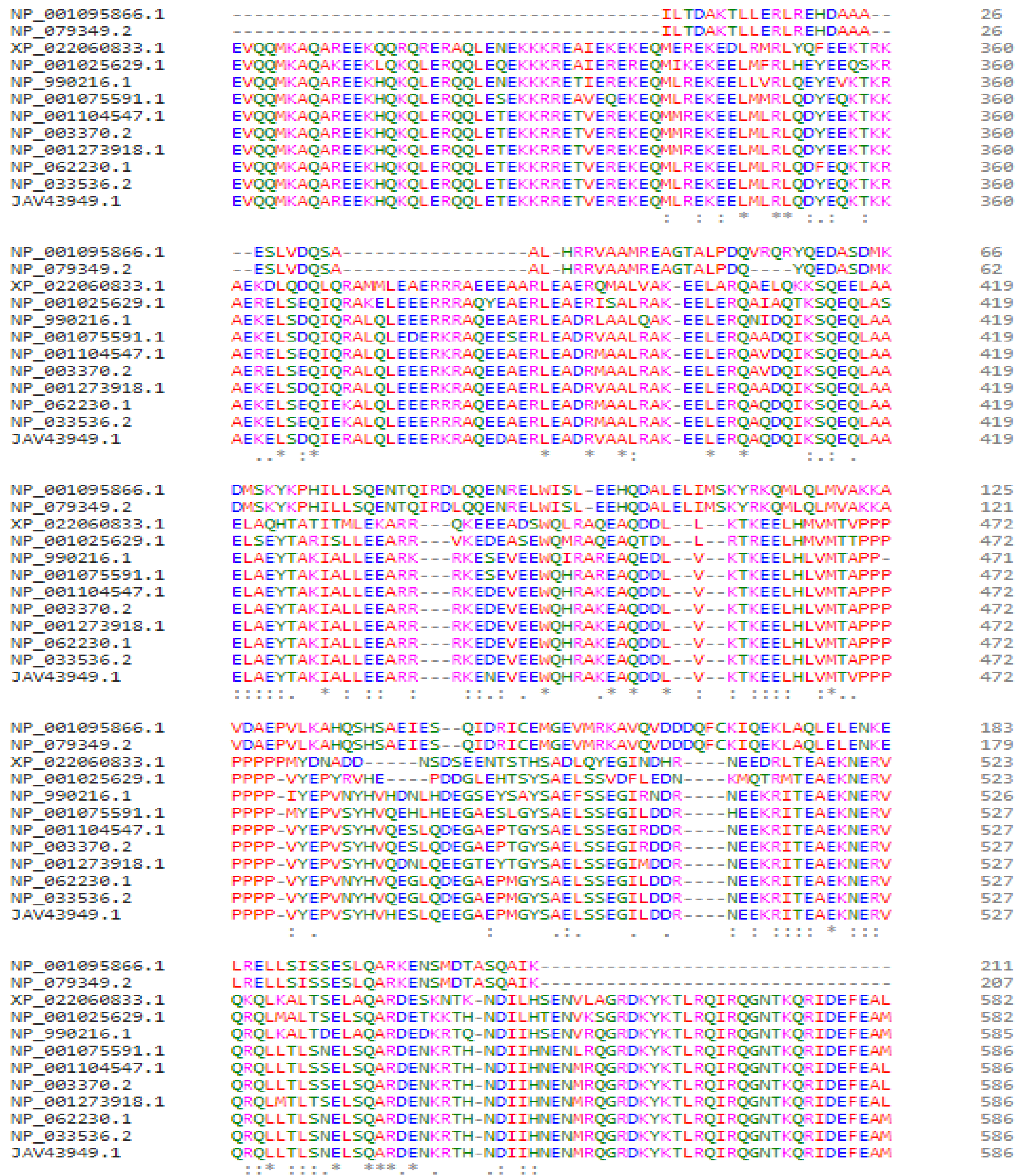

Figure 1 - Clustal Analysis of Ezrin. The latter section of the Ezrin (bottom 10 sequences) clustal alignment demonstrates a high amount of SIKE (top 2 sequences) sequence homology. Dots represent largely conserved residues, while stars represent fully conserved residues across all proteins aligned. Ezrin sequences for several different species were obtained from the PubMed protein database in order to perform this analysis. This sequence from Ezrin, which contains high SIKE homology, was modeled on its own and later docked with SIKE in order to model how Ezrin might interact with SIKE. The same procedure was performed using Beta Catenin, tubulin and Heavy Chain myosin.

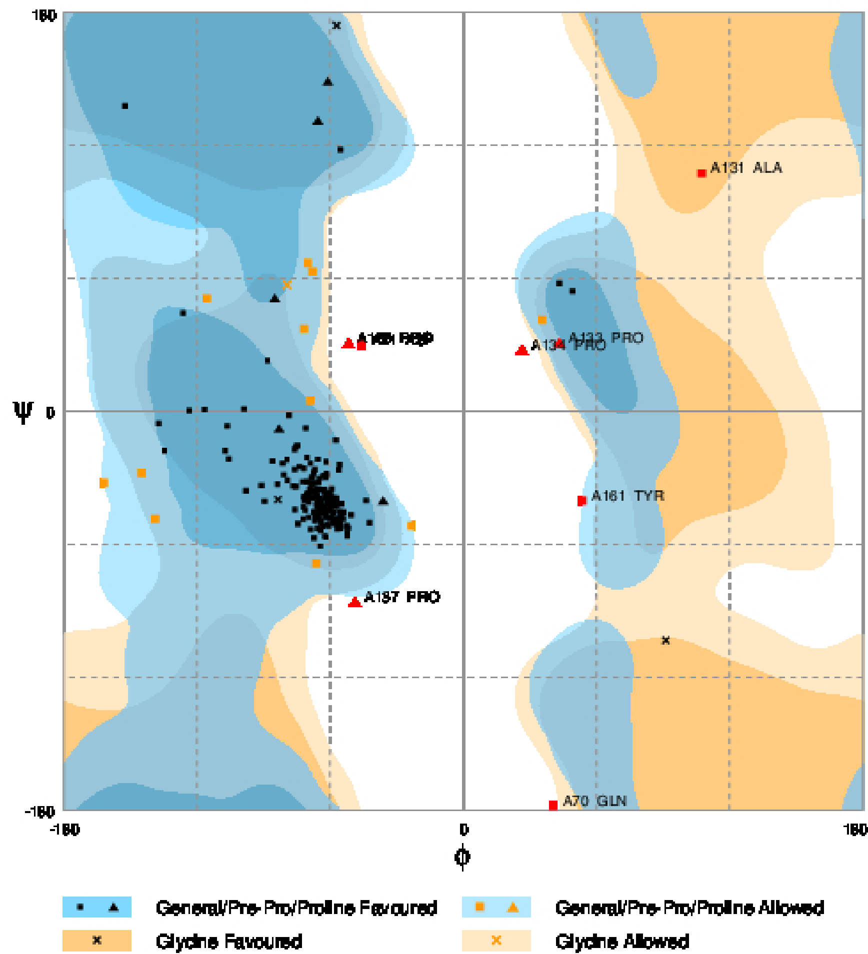

Figure 2 – Ramachandran Plot of Ezrin Model. Ramachandran analysis was performed in order to validate the accuracy of protein section models by examining the energetic favorability of each residue's angles. For the modeling service iTasser, multiple potential models are created, and it is important to confirm that the top model given is in fact the most likely to exist. Comparison between different potential models for Ezrin demonstrated that the top model contained greater than 90% of residues on the favored region, while the second most likely model contained only 70% of residues on the favored regions. Similar results for every other protein model generated confirmed that the top models, which were subsequently chosen for continued use, were, in fact, the most reasonable models generated.

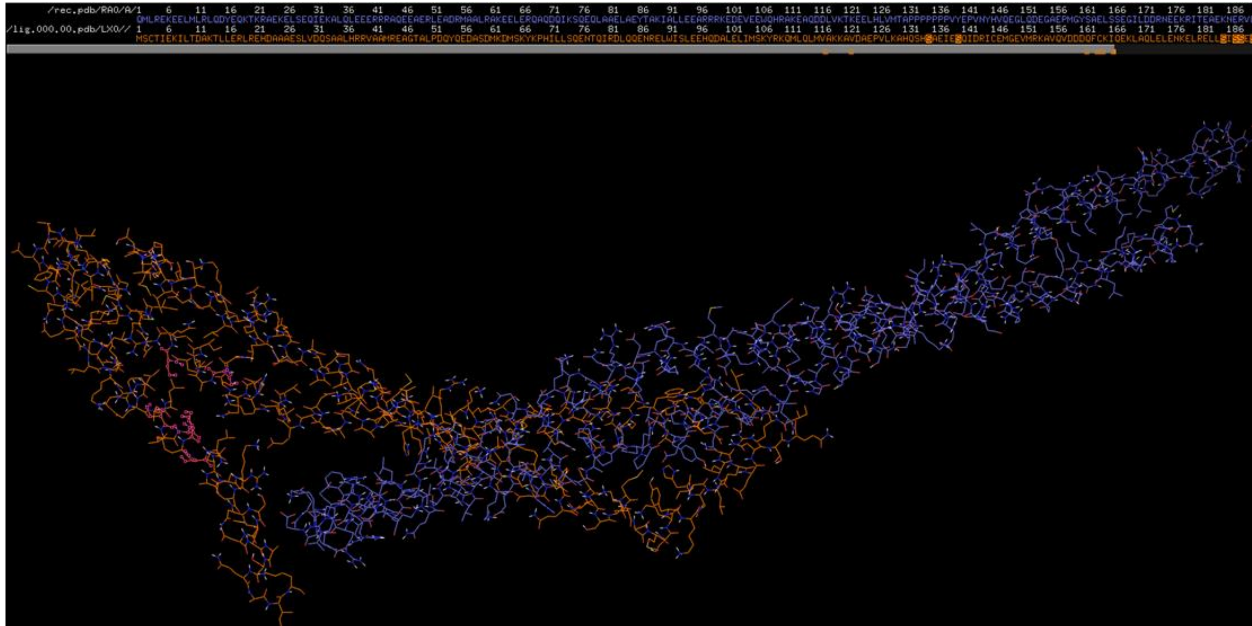

Figure 3- SIKE-Ezrin Interaction Model. A small section of Ezrin (blue) was modeled and docked with SIKE (orange) in order to predict how the proteins might interact. A similar process was performed with Beta Catenin, tubulin and Heavy Chain myosin. Interactions between all four partners and SIKE occurred in the coiled-coil region of SIKE. The phosphorylatable SIKE residues (highlighted in pink) were located very far away from the interaction site in all of the docked models that were tested.

Sike Residue	SIKE-SIKE Dimer	Bcat	Interaction Type	Myosin	Interaction Type	Ezrin	Interaction Type	Tubulin	Interaction Type	Malate	Interaction Type
Y-54		T-15	H Bond			T-130	H Bond				
K-62	D99,E102	T-15/Y-11	H-Bond	E-84	Ionic	T-130	H Bond	P-109		A-236	
Y-67	W91	V-8	Hydrophobic	A-80	Hydrophobic			R-107	H Bond	A-236	Hydrophobic
I-71		S-7	Antagonistic								
Q-75								S-96/S-100	H Bond		
T-78						Q-152	H Bond	N-92	H Bond		
Q-79				R-58/K-177	H Bond	Q-152	H Bond	N-92	H Bond	E-59/D-62/Y-19	H Bond
I-80								T-2/T-9	? Hydrophobic		
R-81						Q-152	H Bond				
L-83										N-27	Antagonistic
Q-84								L-1	Antagonistic		
Q-85								Y-52	H Bond		
E-86				K-168	Ionic						
N-87				K-79	H Bond						
R-88						D-52/Y-145/E-149	Ionic	R-28	ANTAGONISTIC	R-24	ANTAGONISTIC
E-89								R-28	Ionic		
L-90		Y-11/S-7	Antagonistic								
W-91	Y67	N-47	Antagonistic	L-156/E-159/A-161	Hydrophobic	H-146/A-61	Hydrophobic			R-229	Antagonistic
L-94		F-10	Hydrophobic								
E-95		K-49	Ionic					R-117	Ionic	R-229	Ionic
Q-98								K-118		Q-169	
D-99		K-49								Q-169	
E-102		D-56/K-92		Q-150							
I-104		H-22									
Y-108		L-21				T-121/Y-86					
R-109	D52, E56	D-56/K-92	Ionic (weak)	E-139/R-142/E-146	Ionic	E-82	Ionic			D-220	Ionic
K-110										D-214	Ionic
Q-111				E-98	H Bond						
Q-114				E-102/N-106	H Bond						
L-115				R-132/E-103	Antagonistic						
A-121				R-115	Antagonistic						
V-122				Y-114	Antagonistic						
K-195				R-115	ANTAGONISTIC						

Figure 4 - Interaction Analysis. Using the interaction models generated, visual analysis was performed in order to determine which residues were involved in interactions between SIKE and either SIKE itself or each of its four potential partners. The type of interaction occurring was determined based on the residues present in that interaction. SIKE residues that interacted with at least four of the five partners are highlighted in yellow. Most of the commonly occurring SIKE residues were also present in the SIKE-SIKE interactions, suggesting that these interactions are likely to occur in reality.

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