

In Silico Mapping of 14-3-3 ζ and TRAF Protein Interactions

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Introduction

While inflammation ends quickly under normal conditions, its response is prolonged in many chronic diseases resulting in major tissue and organ damage. For example, in rheumatoid arthritis, the cartilage is degraded causing bone erosion. In systemic lupus erythematosus (lupus), cardiac and nervous tissue decays. Autoimmune diseases such as these affect over 23.5 million Americans, and the National Institute of Health has labeled chronic inflammatory diseases as the leading cause of death in the world (National Institute of Health, 2005). However, there are no cures, so seeking new therapies is a favorable solution.

Interleukin-17A (IL-17A) is a cytokine involved in this inflammatory response. It is released by T cells to protect against pathogens and tissue damage (Kuwabara, 2017). Increased IL-17A serum levels have been observed in patients with these diseases showing that IL-17A signaling plays a role in over-inflammation (Van Baarsen, 2014). Due to this unique regulatory role, IL-17A blocking therapies, such as secukinumab (Cosentyx), have been created. However, these therapies have only been effective in psoriasis and no other diseases (Genovese, 2013). The reason current therapies are mostly unsuccessful is that they inhibit the entire IL-17A pathway as well as all of its products. This is unfavorable because in most diseases only specific outputs of the pathway need to be blocked while others are still needed to maintain host defense. Therefore, it is favorable to create a therapy that can block the damaging outputs of a pathway while still retaining the necessary outputs.

Jenna McGowan, a researcher at the University of Toledo Department of Physiology and Pharmacology, has previously reported in the Proceedings of the National Academy of Science the specific molecular mechanisms and outputs of the IL-17A signaling pathway as shown in Figure 1 below. When IL-17A binds to an extracellular receptor, it transduces the signal to TNF

Receptor Associated Factor 5 (TRAF5), and TNF Receptor Associated Factor 6 (TRAF6), which then signals the production of a transcriptional output. TRAF5's output is CXCL-1, and TRAF6 produces IL-6 and IL-8 (McGowan, 2020). CXCL-1 is a chemokine with a range of functions, including inflammation, angiogenesis, tumorigenesis, and wound healing. IL-6 and IL-8 are proinflammatory cytokines (Dhawan, 2002).

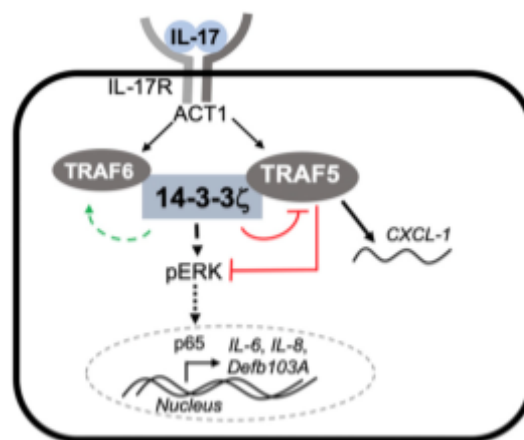


Figure 1. IL-17A signaling pathway (McGowan, 2020).

Further, it was discovered that the 14-3-3 ζ protein is involved in the IL-17A pathway. 14-3-3 ζ is one of seven proteins in the 14-3-3 family. This protein family is highly conserved among eukaryotes and some prokaryotes, meaning that it is evolutionarily important. 14-3-3 ζ specifically is known for its function in many signal transduction pathways, including apoptosis, metabolism, protein transport, and the cell cycle. In the IL-17A pathway specifically, 14-3-3 ζ is a TRAF binding component and a differentiating factor between the activation of TRAF5's output and TRAF6's output. This makes 14-3-3 ζ an attractive target for the selective inhibition of the IL-17A pathway because by blocking the binding of TRAF5 to 14-3-3 ζ I can inhibit the production of CXCL-1 while the TRAF6 pathway, producing IL-6 and IL-8, can remain active, and vice versa. These types of specific therapies are needed to inhibit the damaging outputs of the IL-17A pathway while retaining the products that are necessary to maintain homeostasis.

The purpose of this study is to determine specific binding sites where the TRAF5 protein binds to 14-3-3 ζ and where the TRAF6 protein binds to 14-3-3 ζ . Through mapping the interaction sites between 14-3-3 ζ and TRAF5 and TRAF6 I will know what amino acids to block on the TRAF protein to stop it from binding to 14-3-3 ζ and thus stop the production of CXCL-1

or IL-6 and IL-8. This will help create a future therapy that can selectively inhibit the IL-17A pathway and can be applied to autoimmune diseases, chronic inflammatory diseases, and even the Coronavirus Disease 2019.

Hypothesis

By using an in silico evaluation of the most stable 14-3-3 ζ -TRAF5 complex and the most stable 14-3-3 ζ -TRAF6 complex I will determine a specific binding site where TRAF5 and TRAF6 is most likely to interact with 14-3-3 ζ in the body.

Methods

First, I obtained preliminary files for the 14-3-3 ζ , TRAF5 and TRAF6 protein structures from the RCSB Protein Data Bank (rcsb.org) using PDB structural files 6EF5.pdb, 4GJH.pdb, and 1LB4.pdb respectively.

ZDOCK

Next, I docked the 14-3-3 ζ protein with both Human TRAF5 and Human TRAF6 separately using ZDOCK . Docking is done to bind two interacting proteins together. In this case I am binding 14-3-3 ζ to TRAF5 and TRAF6 separately. This was achieved by inserting 14-3-3 ζ as the receptor and the TRAF protein as the ligand. I then obtained the top 10 out of 2000 predictions for what orientation the 14-3-3 ζ protein will bind to each of the TRAF proteins (Chen, 2003). Each of the 10 predictions is shown as a single trial. ZDOCK was used because it allows the input of binding restrictions at certain amino acids which helps to further refine the models. First I ran this software without any binding restrictions (trials 1-10). Then the procedure was repeated but with an added binding restriction based off of the most stable complex from the

first 10 trials. This helps to create more accurate results by outputting another 10 more refined trials (trials 11-20).

PDB Editor

After docking I used PDB Editor to make the protein files compatible with the later softwares in my procedure. First I inputted the files from ZDOCK into PDB Editor. Then I renumbered all of the amino acids because the output from ZDOCK often overlaps residue numbers which makes identifying specific amino acids in PyMOL difficult. Next, I relabeled all of the TRAF5 protein as Chain A and all of the 14-3-3 ζ protein as Chain B. This is done so that PRODIGY, used last, can better calculate the binding energy and dissociation constant between the TRAF protein and the 14-3-3 ζ protein.

PyMOL

Then, I mutated specific amino acids using PyMOL. This was needed to make the PDB files compatible with a later software in my procedure. 14-3-3 ζ contains multiple phosphoserine residues (SEP), which are a non-standard amino acid and are not compatible with the PRODIGY software. Recent studies have shown that SEP can effectively be mimicked by glutamate without altering the protein's structure (Dissmeyer, 2002). Using PyMOL, I mutated all SEP amino acids in the 14-3-3 ζ molecule to glutamic acid as shown in Figure 2. Residues 511 and 100 were selected and mutated from SEP to glutamate using the mutagenesis function. This step was conducted after docking so that the binding

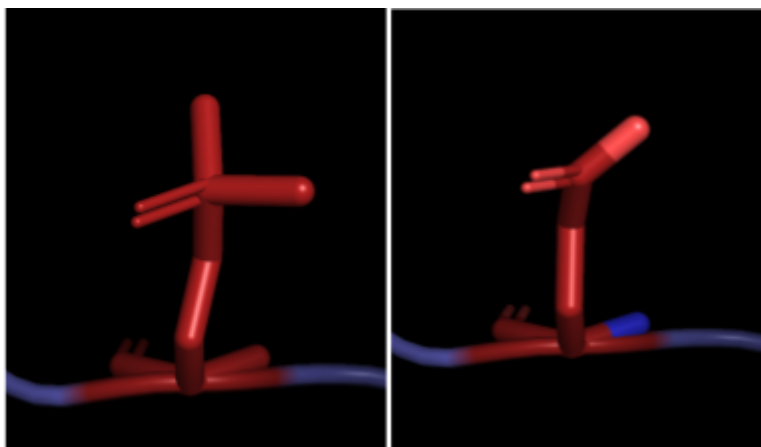


Figure 2. Original phosphoserine (SEP) on left. Mutation to glutamate on right.

predictions would remain accurate to the original molecule and not be affected by the new mutation. PyMOL was the best choice for conducting the mutations because it allows the protein complex to be remodeled locally to the mutation keeping the structure as original as possible. Other softwares remodel the entire protein, which would drastically change the structure of the proteins from what is actually found in the body.

PRODIGY

To further evaluate the interaction quality, all models were imputed into the PRODIGY Webserver, labeling Chain 1 (TRAF) as A and Chain 2 (14-3-3 ζ) as B. This software accurately predicts the binding energy (ΔG) and dissociation constant (K_d) between the Human TRAF molecule and 14-3-3 ζ , which can be used to analyze the complex's stability (Xue, 2016). PRODIGY also outputs a list of interacting residues on the TRAF protein from which I can identify which specific amino acids are interacting between TRAF5 and 14-3-3 ζ and TRAF6 and 14-3-3 ζ .

Results

14-3-3 ζ -TRAF5 Interaction Results

TRAF5					
10 Initial Trials - No Binding			10 Refined Trials - Binded on 479-485		
Trail #	ΔG (kcal mol ⁻¹)	Kd (M) at 25.0 °C	Trail #	ΔG (kcal mol ⁻¹)	Kd (M) at 25.0 °C
1	-9.4	1.30E-07	11	-9.4	1.30E-07
2	-11.9	2.00E-09	12	-8.2	9.20E-07
3	-14.5	2.20E-11	13	-10.1	4.00E-08
4	-8.2	9.20E-07	14	-8.3	7.70E-07
5	-6	3.80E-05	15	-17.5	1.60E-13
6	0	0	16	-4.9	2.60E-04
7	0	0	17	-6.4	2.00E-05
8	0	0	18	-6.4	2.00E-05
9	0	0	19	0	0
10	0	0	20	0	0
Mean	-10	7.81E-06	Mean	-10.7	3.72E-07

Table 1. All mean values are excluding failed trials indicated by zeros or red text.

These results indicate that the amino acid sequence $_{479}\text{LENSKNY}_{485}$ on the TRAF5 molecule is the putative (accepted) binding site when considering the Gibbs free energy (ΔG) and the dissociation constant (Kd). When TRAF5 is binded to 14-3-3 ζ at $_{479}\text{LENSKNY}_{485}$ (trials 11-15 in Table 1), the resulting complex had the lowest mean ΔG of -10.7 kcal/mol. Moreover, when binded at $_{479}\text{LENSKNY}_{485}$, the complex had the lowest minimum ΔG of -17.5 kcal/mol. For the dissociation constant, the binded complex had the lowest mean Kd value of 3.72×10^{-7} and, again, the binded complex also had the lowest minimum Kd value of 1.60×10^{-13} .

In this experiment, there were unsuccessful trials for two different reasons. The first is that the software did not bind the 14-3-3 ζ to the TRAF5 molecule at all. Since there were no interactions between the proteins for these trials, no data could be obtained; this explains the multiple trials with only zeros in Table 1. The second reason that some trials is that the software did not bind TRAF5 to the active site of 14-3-3 ζ . This is shown by Figure 3.

On the complex to the left, the TRAF5 molecule (yellow) is on the side of the 14-3-3 ζ molecule (blue). In this case, the molecules were interacting, just not in the correct spot. These trials are denoted by red text. The correct

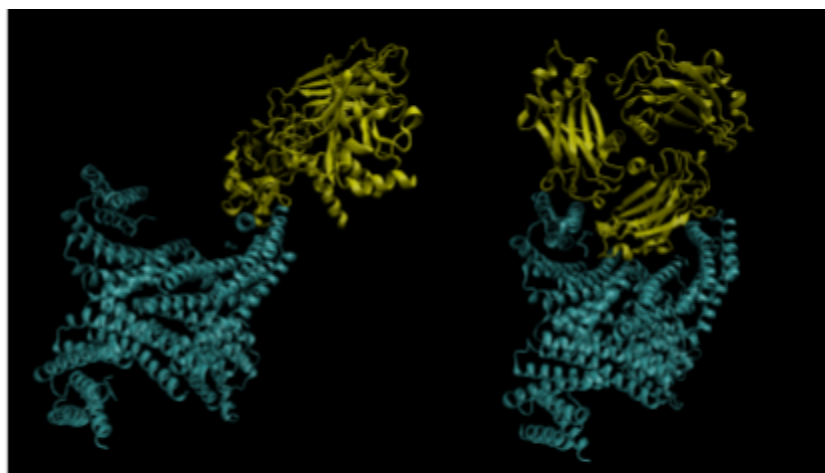


Figure 3. Complex with incorrect binding on left. Complex with the correct binding is on the right. TRAF5 is yellow, 14-3-3 ζ is blue.

complex is shown on the right of Figure 3. Overall, these unsuccessful trials do not detract from the validity because all trials that succeeded showed evidence in support of the hypothesis.

14-3-3 ζ -TRAF6 Interaction Results

TRAF6					
10 Initial Trials - No Binding			10 Refined Trials - Binded on 483-488		
Trail #	ΔG (kcal mol ⁻¹)	Kd (M) at 25.0 °C	Trial #	ΔG (kcal mol ⁻¹)	Kd (M) at 25.0 °C
1	-9.9	5.10E-08	11	-16.3	1.10E-12
2	-13.3	1.70E-10	12	-12.4	7.80E-10
3	-10.1	3.70E-08	13	-19.1	9.50E-15
4	-9.6	8.70E-08	14	-13.6	1.00E-10
5	-13.3	1.80E-10	15	-12.2	1.10E-09
6	-13.1	2.30E-10	16	-11.9	1.80E-09
7	-12.3	8.80E-10	17	-12.8	3.80E-10
8	-13.4	1.40E-10	18	-9.9	5.70E-08
9	-9.8	6.00E-08	19	-15.4	4.70E-12
10	-12.9	3.60E-10	20	-10.6	1.60E-08
Mean	-11.77	2.37E-08	Mean	-13.42	7.72E-09

Table 2. TRAF6 results.

The results indicate that the amino acid sequence $_{483}\text{RQRTFI}_{488}$ on the TRAF6 molecule is interacting with the 14-3-3 ζ molecule. This is because when TRAF6 is binded to 14-3-3 ζ at $_{483}\text{RQRTFI}_{488}$ (trials 11-20 in Table 2) the complex has the lowest mean ΔG of -13.4 kcal/mol and a lower minimum ΔG of -19.1 kcal/mol compared to a minimum of -13.4 kcal/mol. For the dissociation constant, the binded complex had a lower mean value of 7.72×10^{-9} M and the lowest minimum Kd value of 9.5×10^{-15} M.

Statistical Significance

Since ZDOCK outputs only the top 10 of 2000 predicted models there would not be a statistical significance when comparing trials 1-10 to trials 11-20 using a t-test or ANOVA since I would be comparing the top 0.05% of trials to the top 0.05% of trials. But my results from the top 10 trials are significant from the rest of the 2000 trials that ZDOCK predicted, I am just not given data for the rest of the 2000 trials. Therefore, from a bioinformatic perspective, it is safe to assume that the statistical significance of results are taken into account when ZDOCK outputs

the top 10 results, which are significant from the rest of the 2000 predicted models, with a p value < 0.05 since I am comparing the top 0.05% of all predicted models.

Conclusion and Discussion

Overall this study shows that sequence $_{479}\text{LENSKNY}_{485}$ is the binding site for TRAF5 while $_{483}\text{RQRTFI}_{488}$ is the binding site for TRAF6. This is because when binded at these sequences the resulting complexes had the lowest mean and minimum Gibbs free energy values and lowest mean and minimum dissociation constant values. A lower ΔG is favorable because the more negative the ΔG is, the more spontaneous the interaction between the 14-3-3 ζ and TRAF protein is. A lower dissociation constant is also favorable because the closer the K_d is to zero, the more stable the complex will be because it is more likely that the 14-3-3 ζ will remain bound to the TRAF protein and not dissociate into its separate parts. Thus, these results show evidence in support of the hypothesis because the 14-3-3 ζ -TRAF5 complex is most stable when binded at $_{479}\text{LENSKNY}_{485}$ and the 14-3-3 ζ -TRAF6 complex is most stable when binded at $_{483}\text{RQRTFI}_{488}$.

A relevant application of these results is in the Coronavirus Disease 2019 (COVID-19). In this disease there is evidence showing that IL-6 and IL-8 are responsible for the inflammation of respiratory passages, but there is no evidence of CXCL-1 involvement (Magro, 2020). If a current IL-17A therapy was used, it would inhibit IL-6 and IL-8 but it would also unnecessarily inhibit CXCL-1 which could negatively affect the host defense. A future therapy for COVID-19 based on this study could stop the binding of TRAF6 to 14-3-3 ζ by blocking $_{483}\text{RQRTFI}_{488}$. This would inhibit IL-6 and IL-8 production but still bind TRAF5 to 14-3-3 ζ , keeping the production of CXCL-1 active.

However, my results still have one limitation, that the bioinformatic models may not be completely accurate. This is because only rigid docking softwares were used. Currently there are no flexible docking softwares that can bind molecules as large as 14-3-3 ζ and the TRAF proteins, therefore I had to rely on results from a rigid docking software. This is a limitation because the molecules are kept in their original shape and are not flexible throughout the binding, while in the body it is known that the molecules would change shape as they bind with other proteins. Due to this, I will have to confirm these results using in-lab studies.

Nevertheless, I was still able to refine the mapping of 14-3-3 ζ and TRAF interactions from almost infinite possibilities to a single one, so the results still provide a clear direction on the putative binding site between 14-3-3 ζ and the TRAF proteins. Now that the specifics of the 14-3-3 ζ and TRAF interactions are known, this information can be used to create a selective IL-17A inhibitor therapy. I now know that we can selectively inhibit the production of the inflammatory cytokines CXCL-1 by blocking amino acids ₄₇₉LENSKNY₄₈₅ on TRAF5 and inhibit IL-6 and IL-8 by blocking amino acids ₄₈₃RQRTFI₄₈₈ on TRAF6. This therapy would be used to treat autoimmune diseases such as rheumatoid arthritis and lupus, as well as chronic inflammatory diseases, which have been unsuccessful with current global IL-17A inhibitors.

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