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Comparative Analysis of the Binding Interactions Between Mst1-Sav1 and Mst2-Sav1 in the Cellular Apoptotic Pathway

Tanusree Mookherjee
Research Scholar,
Department of Biochemistry and Biophysics, University of Kalyani, Kalyani -741235, Nadia, West Bengal, India
Email- tanusreebcbp18@klyuniv.ac.in

Angshuman Bagchi*
Associate Professor of Biochemistry and Coordinator of Bioinformatics Center, Department of Biochemistry and Biophysics, University of Kalyani, Kalyani -741235, Nadia, West Bengal, India
Email- angshumanb@gmail.com

Rita Ghosh*
Dean, Faculty of Science and Chairperson of Bioinformatics Division, Department of Biochemistry and Biophysics, University of Kalyani, Kalyani - 741235, Nadia, West Bengal, India
Email: ritadg2001@yahoo.co.in;

***Abstract*: Cell multiplication and apoptosis are important for tissue homeostasis and organogenesis. The maintenance of organogenesis is mediated by the Mst1/2 pathway, a unique, multidisciplinary pathway. The proteins Mst1and Mst2 bind to its scaffold protein, Sav1, at their SARAH domain, an interaction which is crucial for their catalytic activation. Dephosphorylated Mst1/2 proteins can promote cell proliferation, while its activation promotes apoptosis. Dysfunction in cellular apoptosis can lead to the onset of cancers, so, the Mst proteins also have a significant role in cancer. It is essential to analyze the molecular interaction of SARAH mediated binding between Mst proteins with Sav1, as this binding is crucial for their catalytic activation. The interactions between Mst2 and Sav1 is already documented, in this study, we have built the three dimensional complex of Mst1 and Sav1 by molecular docking simulations and compared the patterns of binding of Mst1-Sav1 complexes with Mst2-Sav1 complex. Comparison of the binding interactions in the aforementioned complexes revealed the natures of the important amino acid residues. These amino acid residues may therefore be used in future mutational studies to identify their roles in the apoptotic pathway. This is the first such report to reveal the molecular basis of the involvements of Mst1-Sav1 interactions in cellular apoptosis.**

Keywords: Mst pathway, SARAH domain, Sav1, cellular apoptosis, molecular docking

I.INTRODUCTION

Cellular multiplication and apoptosis have a prime role in tissue homeostasis and organogenesis (1). It is maintained by cell signaling pathways. One of such cell signaling pathways is Mst1/2 pathway or mammalian sterile 20 kinase pathway that maintains cell differentiation and organogenesis; it balances cell proliferation and apoptosis through tight regulation (2). The mammalian sterile 20 kinase 1 and 2 (Mst1/2) proteins are the products of stk4 and stk3 genes respectively, that belong to the serine threonine kinase protein family, the GCK-II subfamily protein in particular (3-4). These two proteins are structurally similar, although, there are significant differences. They are 487 and 491 amino acids long respectively, having a kinase domain and SARAH domain. The SARAH domain is named after SAlvador, RAssf and Hippo protein. The helical SARAH domain spans almost 50 residues (5-7). It facilitates the homo and hetero-dimerization of the relevant proteins. The Salvador protein (Sav1) also has the SARAH domain and it heterodimerizes with Mst1/2 via this domain (8). This binding facilitates the activation of Mst1/2 proteins, though they can also be activated by auto-phosphorylation. The binding of Sav1 to Mst1/2 can elicit apoptotic response in cells; activated Mst1/2 can phosphorylate other proteins to inhibit cell proliferation and signal apoptosis, which if left uncontrolled give rise to cancer (9-16). The absence of this interaction leads to loss of function of oncogenic proteins like, Yes associated Protein (Yap) by phosphorylation (17). When Mst1/2 pathway is switched off, Yap is dephophorylated, it can then enter into the nucleus to activate cell proliferation (18-20); whereas, when Mst1 is cleaved by caspase its catalytic fragment translocates to nucleus to phosphorylate histone2B that results in DNA fragmentation leading to apoptosis (21-23). Thus, SARAH domain-mediated binding is essential and crucial for regulating carcinogenesis (24). It is therefore, important to

know the interaction of Mst1 and Mst2 with Sav1 to understand the molecular mechanism of their activation. Though, both the members, Mst1 and Mst2 are involved in the same pathway, their modes of activation are believed to be different. While, interaction between Mst2 - Sav1 has been partially elucidated from X-ray crystallography of the complex, there are no report on the interaction between Mst1 and Sav1. In this work using bioinformatics tools we have analyzed in-silico, the binding of Mst1 with Sav1. It has been reported that two double mutations within the SARAH domain of Sav1 can alter the modes of the interactions. Structural analysis of participating amino acids and two important insertion mutations were done to compare the result with Mst2 - Sav1 complex. This is the first report to elucidate the molecular basis of the binding interactions between Mst1 and Sav1.

II. MATERIALS AND METHOD

A. Sequence analyses and molecular modeling of Mst1, Mst2 and sav1 and Molecular Modeling of the proteins:
The amino acid sequences of Mst1,Mst2 and Sav1 of human were extracted from the Uniprot database (25) with the Accession numbers Q13043,Q13188 and Q9H4B6 respectively. As we are interested in the interactions of the SARAH domains so we took only the sequences of the SARAH domains of all the proteins. The amino acid sequences of the SARAH domains of the proteins are presented below:

Mst1:433YEFLKSWTVEDLQKRLLALDPMMEQEIEEIRQKYQSKRQP ILDAIEAK480

Mst2:437ILKWELFQLADLDTYQGMLKLLFMKELEQIVKMYEAYRQ ALLTELENR484

Sav1:321ILKWELFQLADLDTYQGMLKLLFMKELEQIVKMYEAYRQ ALLTELENR368

The amino acid sequences of Mst1 and Mst2 were aligned together and the percentage of sequence identity was found to be 66.67.
The three dimensional structures of Mst1, Mst2 and Sav1 are available in the Protein Data Bank (PDB) (26) bearing the following pdb ids:
For Mst1: 4NR2 & 4OH8 spanning the amino acid residues 432-480 i.e., the SARAH domain
For Mst2 and Sav1: 6AO5
However, there are some missing atoms in some of the amino acid residues in 4NR2. On the other hand, 4OH8 is a complex of Mst1 and RASSF5. Our aim was to analyze and compare the binding interactions of Mst1 SARAH domain with Sav1 with those of Mst2-Sav1. Therefore, as the starting structure to analyze the binding interactions, we would require a free full chain structure of the SARAH domain of Mst1. Thus, we built the three dimensional structure of the independent SARAH domain of Mst1 with the help of RaptorX (27) and Phyre2 (28) servers. We used both the tools to compare and check the structural qualities of the modeled domain. This was done so that we could obtain a comprehensive result. However, both the servers generated identical structures as observed from the structural alignments of the backbone atoms of the amino acid residues of the SARAH domain of Mst1 protein. The root mean squared deviation (RMSD) between the positions of the backbone atoms was 0Å. Therefore, any of the structures may be chosen for further analysis. However, we used the model of the SARAH domain of Mst1 as obtained from RaptorX.

B. Refinements of the modeled structure: The modeled structure of the SARAH domain of Mst1 was subjected to energy minimizations to remove any unwanted steric clashes. The energy minimizations were performed in the DS2.5 software suite. For minimizations, the steepest descent algorithm was used with the help of CHARMM force-fields. The stereo-chemical qualities of the model were checked using the SAVES server and Ramachandran plots (29) were drawn. No amino acid residues were found to be present in the disallowed regions of the Ramachandran plots.

C. Docking of the SARAH domains of Mst1 and Sav1: To study the binding interactions between the domains, we performed molecular docking simulations using Z-dock (30). Z-dock uses a rigid-body docking method with the help of the Genetic Algorithm (GA). We used the default values of the GA parameters. Since no residue information was available previously for the binding of Mst1 and Sav1, we used a blind docking procedure to generate the docked complex. The stereo-chemical qualities of the docked complexes were again checked by the SAVES server after energy minimizations. We analyzed the binding interactions of the top10 docked complexes as obtained from Z-dock.
D. Analysis of the docked complexes: The energy minimized structures of the docked complexes were analyzed to find

the amino acid residues involved in binding. We also calculated the residue wise binding interactions using the DS2.5 software suite.

E. Comparison of protein structural stabilities: The stabilities of the protein domains before and after docking were checked by the CABS-flex2.0 server (31). This was performed to determine the flexibilities of the amino acid residues in the free and in bound conditions as there was no information available to identify the binding residues.

F. Introduction of double mutation in Sav1 to check the binding free energy:

In order to study the effects of the mutations, we performed in-silico mutagenesis of the Sav1 using the Build Mutant module of DS2.5. The mutants so generated were subjected to energy minimizations following the same protocol as mentioned in the section B. The refined models of the Sav1 mutants, obtained after energy minimizations, were used for docking with Mst1. The docked complexes having the highest binding free energy values were used further for analysis.

III. RESULTS AND DISCUSSION:

A. Sequence Analysis of Mst1, Mst2, and Sav1:

The SARAH domains of both Mst1 and Mst2 consist of 48 amino acid residues and are similar, bearing 66.67% identical amino acids (Figure1). Despite being similar and having functional overlap, it was observed from gene knockout experiments that while, Mst1 null mice were immuno-compromised, Mst2 null mice exhibited no developmental or immunological defects (32). The residue level binding interactions of Mst2 with Sav1 have previously been described. The binding between Mst2 and Sav1 is mediated through their interaction at the SARAH domains was already demonstrated by Bae et al (33). Therefore, we performed a pair-wise comparison of the amino acid sequences of the SARAH domains of Mst1 and Mst2 so that we could identify the amino acid residues of the SARAH domain of Mst1 that matched with those of Mst2. The amino acid residues of SARAH domain of Mst2 that are involved in binding the SARAH domain of Sav1 were found to be mostly conserved in the SARAH domain of Mst1 as well. These amino acid residues are marked in red and presented in bold font in the sequence alignment (Figure1).

Mst1:YEFLKSWTVEDLQKRLLALDPMMEQE**I**EEI**R**Q**KY**QS**K**RQPIL
DAI

Mst2:FDFLKNLSLEELQMRLKALDPMMERE**I**EEL**R**Q**RY**TA**K**RQPIL
DAM

Figure1: Pair-wise comparison of the amino acid sequences of the SARAH domains of Mst1 and Mst2. The amino acid residues involved in binding interactions were marked in red and presented in bold font.

B. Molecular Modeling and model analysis:

In order to study the binding interactions between the SARAH domain of Mst1 with that of Sav1, we generated the three dimensional structure of the SARAH domain of Mst1 by RaptorX server. The modeled SARAH domain of Mst1 was found to be helical. None of the amino acid residues were found to be present in the disallowed regions of the Ramachandran plot. For Sav1, we used the crystal structure 6AO5 which is a complex of Mst2 and Sav1. We extracted the three dimensional structure of the SARAH domain of Sav1 from 6AO5. The SARAH domain of Sav1 was found to consist of helical regions as well. The structures are presented in figure 2. Interestingly, both the proteins were found to belong to the family of all helix domains. The helical regions in the proteins would help them to stack together to generate a suitable protein-protein binding interface necessary to exert their functions.

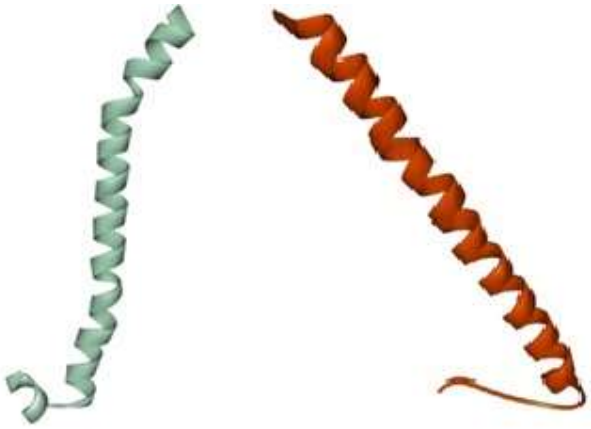

Figure2: Cartoon representations of the SARAH domains of Mst1 and Sav1. Mst1 was presented in green while Sav1 was shown in brown.

C. Analysis of the Mst1-Sav1 docked complexes:

In order to analyze the binding interactions between Mst1 and Sav1, we performed molecular docking simulations using the three dimensional structures of the molecules. For docking simulations, we used the Zdock server. Zdock produced ten docked poses of

Mst1-Sav1 complex. The stereo-chemical qualities of the docked poses were analyzed using SAVES server. None of the amino acid residues of either Mst1 or Sav1 were present in the disallowed regions of the Ramachandran plots. We then calculated the total binding free energy values of the complexes and ranked them accordingly. As per our analysis of the docked complexes we found that the complex 8 had the best binding free energy value followed by complex 3 and 1 (table1). We used these three complexes for further analysis. In order to identify the modes of the binding interactions, we calculated the hydrogen bonding profiles of the complexes (table2). Apart from hydrogen bonding there were hydrophobic interactions between the amino acid residues of Mst1 and Sav1. We analyzed the binding interfaces of the top three docked complexes of Mst1-Sav1 as obtained from Zdock. The best docking pose, viz., the complex 8 was shown in figure 3.

Table1: Details of the binding free energy values of the docked complexes

Serial no of the docking pose obtained from Zdock	**Binding free energy (kJ/mol)**
1	-134.84
3	-144.06
8	-147.65

Table2: Details of the hydrogen bonding interactions in the complexes

Serial no of the docking pose obtained from Zdock	**Interacting amino acid residues of Mst1**	**Interacting amino acid residues of Sav1**
1	Arg447	Phe312, Phe343
3	Arg447, Tyr466	Asp331, Phe343
8	Tyr433, Arg447, Tyr466, Gln467, Lys469, Arg470	Lys323, Leu326, Ile350, Val351, Gln355, Glu366

D. Comparison of the binding interactions of Mst1-Sav1 with those of Mst2-Sav1:

The crystal structure of Mst2-Sav1 complex was obtained from PDB (PDB Code: 6AO5). We again analyzed the binding interface of the Mst2-Sav1 complex in order to make a comparison between the distributions of amino acid residues in the binding interfaces of Mst1-Sav1 and Mst2-Sav1 complexes. We could identify the following amino acid residues of Sav1, viz., Trp324, Phe327, Tyr335, Leu339, Leu342, Phe343, Glu346, Tyr357, Arg358, Leu361, Leu365 and Arg368 to be involved in the binding interactions in Mst1-Sav1 and Mst2-Sav1 complexes. The binding interface amino acid residues of Sav1 were found to be mostly hydrophobic in nature. The amino acid residues Trp324, Phe327, Leu339, Leu342, Phe343, Leu361, and Leu365 of Sav1 were found to undergo hydrophobic interactions with the following amino acid residues of Mst1 and Mst2

Mst1: Phe435, Leu436, Leu45, Met454, Met455, Ile459,

Mst2: Phe439, Leu440, Leu455, Met458, Met459, Ile463

It is also to be noted that the side chain of the amino acid residue Ile459 of Mst1 and was found to be stacked on to the C-alpha backbones Glu346 and Arg358 of SARAH domain of Sav1. It was evident from other studies (SARAH domain-mediated Mst2-RassF dimeric interactions) that SARAH domain favors hydrophobic interactions. These common amino acid residues may therefore be considered to have potential biological significances in exerting the functionalities of the protein complexes. The side chain the amino acid Arg463 was found to form an ion pair with the side chain of Glu346 of Sav1.

E. Analysis of protein structure stability by CABSflex 2.0:

CABSflex2.0,is an online server (http://biocomp.chem.uw.edu.pl/CABSflex2) providing the measure of fluctuation of the amino acids during interaction with other proteins. Here we made a comparison of complexes, 1, 3, and 8 with 6AO5. It was revealed from the analysis that the amino acid residues from 335 to 371 of the SARAH domain of Sav1 would have the lowest RMSF value in the Mst2-Sav1 complex (6AO5). For Mst1-Sav1 complexes the following regions of SARAH domain of Mst1 were found to be the most stable:

Complex1: Amino acid residues spanning 442 to 467,

Complex3: Amino acid residues spanning 442 to 462

Complex8: Amino acid residues spanning 442 to 472.

For the Mst2 SARAH domain, the amino acids spanning between the region 450 to 470 was found to have the lowest fluctuations. All these results would indicate that the patterns of binding interactions were more or less conserved in Mst1-Sav1 and Mst2-Sav1 complexes.

F. Introduction of double mutation in Sav1 to check the binding free energy

Mutations at the positions Glu346 (E346A) and Arg358 (R358A) of Sav1 were introduced by DS 2.5 and the structures were analyzed by PROCHECK and energy minimized in DS 2.5. The structures of mutant Sav1 and Mst1 were again docked in Zdock and the best model as per the highest binding free energy value was selected. However, the mutants of Sav1 were found to form weaker complexes than the wild-type Sav1. The amino acid residues Ile459 and Arg463 of Mst1 were found to be absent from the binding interface. Therefore, the mutations in the amino acid residues of Sav1, viz., E346A and R358A would tend to abolish a major component of the binding interactions between Mst1 and Sav1. The amino acid residues Ile459 and Arg463 were found to be belonging to the region having the least structural fluctuations in the RMSF analysis. Therefore, this binding interaction between Mst1 and Sav1 may be considered to have a significant role in forming the Mst1-Sav1 complex. The similar residues in Mst2 are Ile463 and Arg467. These amino acid residues of Mst2 were also found to be present in the region of least fluctuations as detected from the RMSF analysis. Therefore, these amino acid residues of Mst2 may also be considered to play a pivotal role in forming the Mst2-Sav1 complex. The mutational study of Sav1 would therefore point towards the importance of the amino acid residues of Mst1 and Mst2 to generate the complex. Interestingly, it is also to be noted that the orientations of the SARAH domains in Mst1 and Sav1 got completely changed (Figure3).

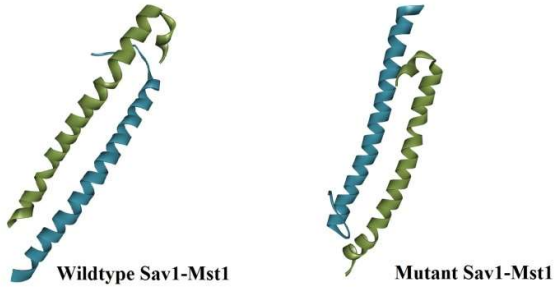

Figure3: The relative dispositions of the SARAH domains in wildtype and mutatnt Sav1-Mst1 complexes. Mst1 was presented in green while Sav1 was shown in blue.

The mutations brought in a drastic conformational change in the Sav1-Mst1 protein complex. Such change in orientations of the complexes might be considered for the loss in functionality of the complex.

Further wet lab experiments may be performed to check the binding efficacies of these amino acid residues of Mst1 and Mst2 to predict the molecular mechanism of the process.

IV. CONCLUSION

Mst1 and Mst2, the two important proteins necessary for the growth and development of mammalian species, also have a role in the development of cancer. In spite of the similarity in their function, Mst1 and Mst2 have some major differences in their functioning. Both Mst1 and Mst2 are known to exhibit their functionalities by binding with Sav1. However, the residue level details of the binding interactions between Mst1 and Sav1 were yet to be identified. We, for the first time, predicted the amino acid residues involved in forming the Mst1-Sav1 and Mst2-Sav1 complexes, to indicate the commonalities between the binding interactions. We also predicted the important amino acid residues of Mst1 and Mst2 which could be thought to be playing pivotal roles in forming the complexes. The mutation study also provided the knowledge about the change in the orientation of Mst1-Sav1 helices. From parallel (the wild type docked complex) to anti-parallel (the mutant docked complex) orientation of two SARAH domains could possibly cause steric hindrance that can be discouraging for the activation of Mst1 kinases. Therefore, our prediction analysis could help to facilitate future mutational experiments for the analysis of the binding modes of the interactions and would further help to

elucidate the hitherto unknown molecular mechanism of the biochemical pathways involving these proteins.

CONFLICT OF INTEREST: There exist no conflicts of interests among the authors

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