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Binding Interactions between MIT Domains and ESCRT-III Proteins

Matthew Miller
Department of Biochemistry
The University of Utah
Salt Lake City, Utah

Faculty Advisors: Dr. Wesley Sundquist and Dr. Dawn Wenzel

Abstract

The ESCRT-III (Endosomal Sorting Complexes Required for Transport) proteins play crucial roles in controlling abscission (the process of separating daughter cells in cytokinesis) (Figure 1) and other cellular processes such as HIV budding that involve membrane fission. The ESCRT pathway is a crucial part of cellular function in all eukaryotic organisms(1). ESCRT-III proteins provide the constricting force required for membrane fission, and they recruit essential cofactors that contain Microtubule Interacting and Trafficking (MIT) domains. An example of a crucial role that the ESCRT machinery plays is that the machinery reseals the nuclear envelope during anaphase(1). We have studied how the ESCRT pathway functions by performing binding experiments with the greater than 20 known MIT domains that interact with the ESCRT pathway, which will provide a knowledge of the processes in which these proteins function. This will help us understand the process by which HIV takes advantage of the body's ESCRT machinery that facilitates cell division, as well as how cell division occurs. We used biochemical techniques such as protein expression, protein purification, size exclusion chromatography, ion exchange chromatography and fluorescence polarization to identify how the MIT domains bind with the various Microtubule Interacting Motifs (MIMs) of the ESCRT-III pathway. MIMs are the C-terminal tails of ESCRT-III proteins that facilitate interactions between the ESCRT machinery and MIT domain-containing proteins.We discovered that MIT domain-containing proteins have very different binding specifities. An example of this would be Katanin, which only interacts with CHMP3 in comparsion with MITD1 that has a much broader binding specificity and interacts with CHMP1A, CHMP1B, CHMP2A, CHMP2B, CHMP3, CHMP6, and IST1. The knowledge that Katanin only interacts with CHMP3 raises a lot of new questions such as how these proteins interact, and why does it only interact with CHMP3.

Keywords: Binding, MIT Domains, Proteins

1. Background

The ESCRT pathway performs a variety of membrane remodeling functions such as MVB (Multi Vesicular Bodies) microvesicle formation, exosome formation, enveloped virus budding, and abcission(2) (Figure 1). ESCRT proteins polymerize within the bud neck of membranes to facilitate the pinching off of the budneck(3) (Figure 2). There are 12 ESCRT-III proteins: CHMP1A, CHMP1B, CHMP2A, CHMP2B, CHMP3, CHMP4A, CHMP4B, CHMP4C, CHMP5, CHMP6, CHMP7 and IST1.The MIT (Microtubule Interacting and Trafficking) domains bind to C-terminal sequences of the ESCRT-III protein or MIM (Microtubule Interacting Motif).

There are approximately 20 MIT domain-containing proteins that regulate function within the ESCRT Pathway (Figure 3). MIT and MIM interactions ensure recruitment of various proteins, including ATPases, microtubule severing proteases, deubiquitinases, and kinases(4) (Figure 4).

There are five main binding modes for MIT domains to bind MIMs that have been identified thus far (Figure 5). The different binding modes indicate that the MIT domains and the MIMs don't always interact in the same way, but bind to different surfaces on the MIT domain (Figure 5). Some of the ESCRT-III proteins contain two different MIMs such as IST1, which has a MIM1 and a MIM2, and it is not entirely clear how many other ESCRT-III proteins contain

multiple MIMs. This is of particular interest because of avidity, which means that the multiple MIMs could potentially allow for tighter binding, or could allow for multiple interactions with different MIT domains to occur concurrently.

Spartin is considered to be a particularly interesting protein because it is affected by certain hereditary spastic paraplegias (HSPs) such as Troyer syndrome, which leads to the absence of cellular Spartin[7]. Dysfunctional Spartin results in muscular dystrophy and on a cellular level, cytokinesis is impaired. Although the exact function of both Spartin and Spastin is not yet fully understood, the two proteins seem to have similar roles within cell division and removal of either protein results in an inability of the cell to divide properly[7].

Fewer than half of the known MIT domains have been studied and more MIT domains continue to be discovered during the study of the ESCRT pathway. An example of this is RPS6KC1, which we are currently studying. There is very little knowledge about the role of this kinase in cell division. In general, kinases are a type of enzyme that catalyzes phosphorylation. They also regulate activity of other enzymes. RPS6KC1 could be an interesting and completely unknown regulator of the ESCRT pathway. We hope that as we study the interactions of the ESCRT pathway we will learn more about cell division.

Some examples of proteins that we studied that contain MIT domains include MITD1, which acts to coordinate abscission with other cellular events. We also study VPS4A, VPS4B, and RPS6KC1. The ESCRT pathway and the interaction between MIMs and MIT domains are not yet fully understood. Our goal is to study these the reactions of ESCRT-III proteins and MIT domains so that we can understand why or if all of these proteins are involved in cell division, which proteins interact with each other and the reason for each protein's binding specificity.

A very important protein in the ESCRT pathway is VPS4. VPS4 is an AAA-ATPase that contains an MIT domain (Figure 4). VPS4 invests the energy from ATP hydrolysis into energy to disassemble the ESCRT-III filament and thereby recycles its individual subunits back to the cytoplasm. VPS4 also dissociates after ESCRT-III disassembly. All humans have two VPS4 proteins (A and B) that play a role in cell division. The roles of VPS4A and B seem redundant, owing to the fact that they are extremely structurally similar and most organisms only contain one VPS4 protein. On the other hand, no one has examined all of the VPS4 proteins binding specificities for ESCRT-III proteins. This is important because it has been established that without VPS4B, cell division cannot occur.

Another protein that plays a crucial role in abcission is MITD1. MITD1 does not have a particular enzyme function, but without its presence during abcission, cytokinesis fails to occur. MITD1 depletion results in cytokinesis failure due to both midbody instability and abscission arrest, which means that the cell fails to divide if MITD1 is removed from the cell[5].

Studying the interactions of the ESCRT-III pathway has already yielded very useful information about proteins such as Katanin and Spastin. Katanin is a protein that is essential because it severs and disassembles microtubules to tubulin dimers[6]. Katanin functions as an ATPase and therefore requires the use of ATP to disassemble microtubules. It is of particular interest that Katanin only binds to CHMP3. We postulate that this occurs because CHMP3 is proximal to the site where microtubules are located and therefore binding to CHMP3 helps facilitate microtubule disassembly.

Another protein that has particularly interesting binding specificity is Spastin. Spastin has been shown to bind to both CHMP1B and IST1. It is very surprising that it binds to CHMP1B and not CHMP1A because the two proteins are very similar structurally, but extremely different in terms of binding specificity. We think this may have something to do with the fact that Spastin's main function is to sever microtubules and thus binding to CHMP1B and IST1 may help facilitate this function[8].

2. Methods

Here we studied the specificity of VPS4B, Spartin, MITD1 and RPS6KC1 for ESCRT-III MIMs. The process consisted of two main steps: protein purification and fluorescence polarization.

2.1 Protein Purification:

An example of the process we used to purify the MIT domain containing proteins was the purification of VPS4B. We used a similar process to purify the other proteins. We purified the MIT domain from VPS4B and tested it for binding against all MIMs (Figure 6). The MIT domain of VPS4B was expressed and purified as a GST-fusion (Glutathione S Transferase) protein. We instead used a HIS (hexahistidine) tag for the purification of Spartin.

We then lysed the cells and purified the protein using affinity chromatography, which allowed us to isolate the MIT domain containing protein based on its GST tag. Then we used anion ion exchange chromatography, which allowed us to isolate our protein based on charge. We eluted our protein using a high salt gradient. We also used size exclusion

chromatography, which relies on isolating our protein based on its size of about 10 kDa. We performed a very similar process in order to purify our ESCRT-III proteins.

2.2 Fluorescence Anisotropy:

We then measured how well VPS4B binds with fluorescently-labeled peptides encompassing MIM's from ESCRT-III proteins. We performed this testing using fluorescence polarization of mixtures of VPS4B and a specific MIM to check the binding. We isolated our protein and performed binding experiments with VPS4B, MITD1 and MITD1 with the different C-terminal tails of the ESCRT-III pathway to understand how they interact during abscission (Figure 1).

3. Results

As a collective effort we have measured Kds for more than half of the MIT-MIM interactions (Figure 6). This has been very beneficial because we are now able to compare different proteins to understand different reactions in the ESCRT-III pathway. VPS4A and VPS4B's binding specificities were very different: VPS4A binds CHMP1A with a KD of 16 µM vs. 80 µM for VPS4B. The same can be said for VPS4A and VPS4B's binding of CHMP1B (23 µM vs. 87 µM) (Figure 3). VPS4A is also a much tighter binder of CHMP2A (3 µM vs 18 µM) and CHMP2B (37 µM vs. 95 µM). VPS4A also binds CHMP6 whereas VPS4B shows no detectable binding. It is also of interest to note that both VPS4A and VPS4B bind more tightly to MIM2 of IST1. The increased specificity of VPS4B suggests that there are most likely substantial differences in function between VPS4A and VPS4B. This is very surprising because it has been hypothesized that the two proteins were relatively redundant owing to the fact that they have incredibly similar amino acid sequences and very similar structure.

We have also discovered that MITD1 is a very promiscuous binder (Figure 7). MITD1 binds very tightly to CHMP2A (7.5 µM) and less tightly to CHMP1A (24 µM) and CHMP1B (46 µM) (Figure 7). It also binds relatively tightly to CHMP3 (29 µM), CHMP4A (38 µM), and CHMP4B (15 µM). It also binds very tightly to CHMP6 (11 µM). MITD1 only binds the MIM1 of IST1 (0.8 µM). It is of particular interest that MITD1 binds to CHMP4A and CHMP4B as well as CHMP6 because these interactions haven't been seen in previous research. We have also learned very interesting things about the specificity of Spartin because it binds only to IST1 (Figure 8). It is a very tight binder 0.6 µM. It only binds to MIM1, which is of particular interest.

4. Conclusion

Throughout the course of this experiment we measured the KD's (binding constants) for various MIM and MIT interactions by purifying proteins that contain MIT domains and proteins that contain MIMs and performing binding experiments using fluorescence polarization. We then used the KD's we have obtained to learn more about the function of this essential pathway.

Further study of both Spartin's and Spastin's interactions with the ESCRT pathway could potentially help us understand complex genetic diseases such as Troyer's syndrome[8]. Studying Spartin more could help lead to better understanding of neurodegenerative diseases such as hereditary spastic paraplegia and Alzheimer's[7].

From a biological perspective, this knowledge will help us learn how abscission, MVB vesicle formation, exosome formation, shedding microvesicle formation and enveloped virus budding occur. On a broader scale, we hope to be able to use this knowledge to identify new proteins that function with the ESCRT pathway that could help with cancer treatments and treatment of other diseases in the future. We also hope to better understand how HIV hijacks the ESCRT pathway, thus taking advantage of the cell's natural ability to form vesicles. This could be beneficial to improve treatments in the future.

Figure 1

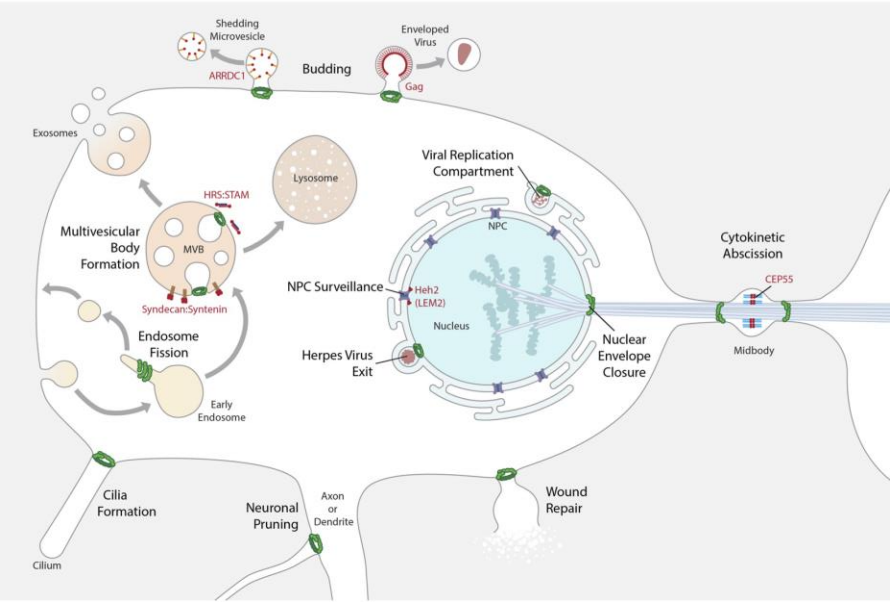

Figure 1.: The ESCRT pathway is involved in many different membrane remodeling processes including abcission and cell division. Image Courtesy: Janet Iwasa

Figure 2

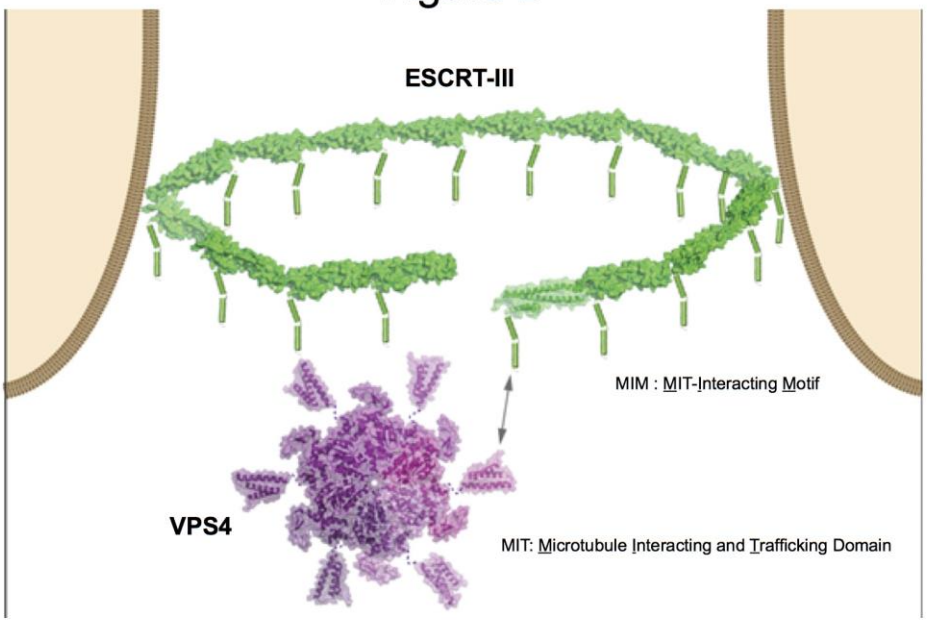

Figure. 2: ESCRT-III proteins play a crucial role in forming a bud neck during cell division. (Figure from McCullough J., Colf, L. and W. I. Sundquist, 2013)

Figure 3

K_D (μM)	VPS4A	VPS4B	MITD1	Spartin	Spastin
C1A	16±3	80±4	24±6	>200	>200
C1B	23±5	87±27	46±5	>200	15±5
C2A	2±1	18±6	7.5±3	>200	>200
C2B	37±3	95±15	>200	>200	>200
C3	>200	>200	29±9	>200	>200
C4A	>200	>200	38±2	>200	>200
C4B	>200	>200	15±1	>200	>200
C4C	>200	>200	>200	>200	>200
C5	>200	>200	>200	>200	>200
C6	54±1	>200	11±4	>200	>200
C7	>200	>200	>200	>200	>200
IST1	0.5±0.2	12±2	0.5±0.1	0.6±0.3	29±4
MIM1	76±11	37±3	8±4	0.4±0.1	2.5±0.6
MIM2	11±2	30±3	38±4	>200	>200

< 2 μM
2 – 20 μM
>20 – 200 μM
> 200 μM

Fig. 3: The MIT domain-containing proteins and their dissociation constants (KDs (μM)) with different ESCRT-III MIMs. The ESCRT-III proteins include CHMP1A-IST1 on the left and the MIT domains are across the top.

5. References

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