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Ph.D. Thesis

Characterization of the dynamics and mechanism of the nuclear import of Drosophila moesin protein

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PUBLICATIONS

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INTRODUCTION

Mechanisms of nuclear transport

Macromolecules no bigger in size than ~60 kDa, small molecules and ions are capable of passive diffusion through the Nuclear Pore Complex (NPC) (Wang and Brattain, 2007). Molecules with molecular weights greater than 60 kDa can enter and exit the nucleus using signal-mediated nuclear transport (Kaffman and O'Shea, 1999).

The process of nuclear import has three main steps: docking, translocation through the pore complex and finally, deposition of the cargo in the nucleus. Inside the nucleus, the cargo-importin complex dissociates and the importin gets transported back into the cytoplasm, where it can take part in further cycles of nuclear import (Kaffman and O'Shea, 1999). The importin recognizes its cargo through a specific sequence of amino acids, called the nuclear localization signal (NLS), which is found on the protein to be imported. Two of the best known NLS motifs are the simian virus 40 large T-antigen NLS (SV40 NLS) and the bipartite NLS of nucleoplasmin. The latter NLS is composed of two shorter clusters (2 and 4 residues) of basic amino acids separated by a 10 amino acids-long spacer (Weis, 1998).

In terms of its main steps, nuclear export process is similar to nuclear import. First, the cargo to be exported into the cytoplasm is recognized through a nuclear export signal (NES) by a soluble export receptor, called exportin. The trimeric complex of cargo-exportin-Ran-GTP is then transported through the NPC into the cytoplasm, where the complex dissociates and the cargo is released (Mattaj and Englmeier, 1998). The best known nuclear export pathway uses a leucine-rich nuclear export signal, which is recognized by the nuclear export receptor Crm1 (also known as Exportin1) (Fornerod et al., 1997).

Regulation of subcellular localization

NLS and NES sequences can target proteins to either the nucleus or the cytoplasm, but the regulation of the subcellular localization of proteins offers few additional ways that fine tune their distribution. Among these regulatory mechanisms is the post-translational modification of the cargo, which can prevent or promote the formation of the cargo-transportin complex. Similarly, the activity of the transportin can also be altered to influence complex formation with the cargo. For example, the cargo-transportin complex can be anchored to an insoluble component by the transportin, preventing the interaction between the complex and the NPC. Another possible way of transport regulation is to modify the NPC itself, which can affect the properties of the transport (Kaffman and O'Shea, 1999; Hung and Link, 2011). An example for the regulation of the subcellular localization of the cargo by post-translational modification is the regulation of the Nuclear factor of activated T cells (NF-AT) protein by phosphorylation. Under normal conditions, NF-AT is phosphorylated and resides in the cytoplasm in a conformation in which the NES is accessible but the NLS is not. Upon T-cell receptor activation, it is dephosphorylated by calcineurin, which causes a conformational change in NF-AT that exposes the NLS (Zhu and McKeon, 1999).In addition to post-translational modifications, the formation of intermolecular complexes is also a possible way by which nuclear localization can be

achieved or inhibited. In the case of Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), association with its binding partners causes its retention in the cytoplasm (Rothwarf et al., 1998). The Nuclear factor 7 (Xnf7) protein of the African clawed frog, *Xenopus laevis* is a maternally expressed transcription factor that is retained in the cytoplasm of the oocyte up until the midblastula transition phase of development. In this case the cytoplasmic retention is achieved by a 22 amino acids-long cytoplasmic retention domain (CRD) (Li et al., 1994). The retention of proteins in the cytoplasm can be performed by many binding partners or even heat shock proteins, but there are also factors that specialize in this activity. For instance, members in the 14-3-3 protein family constitute such a group of evolutionary conserved regulatory molecules. So far, more than 200 molecules have been shown to act as substrates for 14-3-3 proteins (Fu et al., 2000).

The ERM protein family

The ERM protein family has three paralogs in vertebrates: ezrin, radixin and moesin, while non-vertebrate species have only one ERM protein. In the case of *Drosophila melanogaster*, the single ERM protein coding gene is called *moesin*. ERM proteins display a high degree of structural similarity to each other. They have three distinct domains, an N-terminal, globular FERM (abbreviated form the names Band 4.1, **E**zrin, **R**adixin, **M**oesin) domain, the so called C-ERMAD (C-terminal ERM Association Domain) at the C-terminus, and a flexible alpha-helical domain which connects the two. As a versatile protein binding domain, the FERM domain is responsible for the interaction with numerous membrane associated proteins. The C-terminal domain has two major functions. It is the binding site of F-actin, and secondly, it plays a role in the regulation of the ERM protein itself as it is able to the FERM domain both inter- and intramolecularly. This inactivation through self-binding is enabled by the flexion of the central alpha-helical domain (Fehon et al., 2010).

The activity of ERM proteins is regulated through head-to-tail folding because in their closed state, when the C-ERMAD is bound to the FERM domain, the intramolecular interaction masks the binding sites on the surface of the FERM domain. To achieve the open, active conformation, ERMs must bind phosphatidylinositol 4,5-bisphosphate (PIP2) in a specific binding pocket located on the surface of the FERM domain, which causes the incomplete opening of the molecule. In this partially opened state, a threonine residue in the C-ERMAD becomes accessible and phosphorylated, causing the full and stable unfolding of the molecule (Nakamura et al., 1995; Fievet et al., 2004; Ben-Aissa et al., 2012).

ERM proteins in the nucleus

With new results emerging, it is clear today that the majority of cytoskeletal proteins are not only present in the cytoplasm, where they perform their classic, well-known tasks, but also in the nucleus. Today we know, that actin, one of the best known cytoskeletal proteins, plays important roles in the nucleus by taking part in processes which regulate the activity of RNA polymerases and transcription factors, chromatin remodeling complexes and histone deacetylases. Nuclear actin has also been linked to human diseases, such as cancer, neurodegeneration and myopathies. Today it is also known that the nuclear transport

of actin, due to its essential nuclear functions, is a tightly regulated, active process (Dopie et al., 2012; Kelpsch and Tootle, 2018).

Similar to actin, the presence of ERM proteins in the nucleus has also been reported. Among the earliest observations of this phenomenon was when a 55 kDa, endogenously cleaved fragment of ezrin was shown to localize to the nucleus (more specifically, to the nucleolus) in human cells (Kaul et al., 1999), or when full length ezrin was observed by immunofluorescence in the nuclei of rat Schwann cells (Melendez-Vasquez et al., 2001). Later, moesin was also detected in the nuclei of human lymphocytes (Bergquist et al., 2001), and it was also shown, that exogenously expressed, GFP-tagged radixin localizes to the nucleus. Differential detergent extraction experiments revealed that ezrin and moesin are tightly associated with nuclear components, further supporting the notion that there are certain nuclear functions that cause ERM proteins to be found in the nucleus (Batchelor et al., 2004).

The Drosophila ERM protein, moesin in the nucleus

In *Drosophila melanogaster*, there is only a single representative of the ERM family, the Drosophila moesin protein. Like its vertebrate homologs, Drosophila moesin also localizes to the cell nucleus. It was shown in cultured Drosophila S2 cells and embryos, that moesin localizes to the nucleus in interphase, and that it exhibits a nuclear distribution complementary to the chromatin (Vilmos et al., 2009). Later studies in the fruit fly shed more light on the nuclear functions of Drosophila moesin. Upon blocking the nuclear export of mRNAs, moesin accumulates to high levels in the nucleus. Using the polytene chromosomes of the Drosophila larval salivary gland, it was shown, that moesin localizes to the chromosome puffs, which are special euchromatic regions with extremely high transcriptional activity. In another study, the authors demonstrated that moesin is a constituent of the mRNP particles responsible for the nuclear export of mRNAs, suggesting that the protein also plays a role in mRNA export (Kristó et al., 2017).

The biological significance of nuclear moesin has also been investigated in *Drosophila* (Bajusz et al., 2021). Since complete absence of moesin activity is lethal for the cell, and also because any change to the protein would affect not only nuclear but also essential cytoplasmic functions, the authors created a version of moesin in the fruit fly, that is fused with a nuclear export signal (NES). Flies expressing the moesin-NES mutant protein exhibited slow development, decreased lifespan, egg production and climbing ability, and genitalia rotation in males. The consequences of reduced nuclear moesin levels were evaluated also at the molecular level by an mRNA-Seq analysis. The experiment revealed, that the expression of 371 genes were up- and 315 were downregulated (Bajusz et al., 2021). Among the upregulated genes were three important players in development: *vasa*, *Notch* and *dpp*. This could explain the developmental defects observed in the moesin-NES mutant animals. Another interesting group of genes, that were downregulated in moesin-NES animals were Heat shock protein (HSP) encoding genes, namely *hsp70Aa, hsp70Ab, hsp70Ba, hsp68, hsp26,* and *hsp23*. This result is consistent with the findings published earlier (Kristó et al., 2017), where upon heat-shock, moesin localized to the heat shock puffs in the polytene chromosomes of Drosophila larval salivary glands, indicating that moesin is required for the transcription of heat-shock genes.

AIMS

Actin-binding FERM domain-containing proteins were already observed to be present in the cell nucleus decades ago, and in the case of some of them, the nuclear functions have also been described in sufficient details. However, the mechanism and regulation of their nuclear transport, even in the case of the best characterized proteins, is still barely known. This is despite the fact that the exploration of nuclear transport can be of great help both in understanding the nuclear function of a given protein as well as in its manipulation. In the present study, we aimed to answer the following questions:

1. What is the dynamics of the nuclear import of moesin?
2. It is known, that moesin has a bipartite NLS. Is the NLS evolutionarily conserved? Does conservation reveal other properties of the NLS, such as its regulation?
3. Does phosphorylation play a role in the regulation of the nuclear import of moesin?
4. Can open or closed conformation regulate moesin's import?
5. What other mechanisms regulate the nuclear import of moesin?

RESULTS

Moesin's nuclear import is an active, regulated process

Nuclear FRAP assays were carried out in order to investigate the nuclear import dynamics of the GFP-tagged Drosophila moesin protein. In order to evaluate the extent of unwanted photobleaching during the assay, so called "blind FRAP" experiments were carried out, which showed that there was no significant bleaching during the 40 minutes of the experiment. The FRAP curve obtained for moesin was compared with GFP-labeled actin, which is one of the main interacting partners of moesin, and the dynamics of its nuclear import is already known from the literature (Dopie et al., 2012). The nuclear import FRAP curve of actin represents a very dynamic import, while the curve acquired with moesin is quite flat, indicating a slow and steady influx of the protein into the nucleus.

It has already been known, that moesin accumulates in the nucleus when mRNA export is blocked. We also examined the dynamics of the import under such conditions and found, that by blocking the mRNA export, the nuclear import of moesin becomes more dynamic, however it is still not as strong as that of actin. Based on these results the most plausible explanation is, that under normal conditions the nuclear import of moesin is strongly suppressed, and when the import is induced (by blocking mRNA export), the retention is partially resolved. These experiments also showed us, that the nuclear import of moesin is an active, regulated process, because if the protein is needed in the nucleus in greater quantities, the dynamics of its import can increase.

Drosophila moesin has an evolutionary conserved, bipartite NLS

The NLS of the moesin protein was identified and characterized previously by Ildikó Kristó, Csaba Bajusz and Anikó Szabó in our laboratory. In sum, out of four predicted NLS sites, the RRRK sequence at positions 294-297 proved to be responsible for nuclear entry. The motif turned out to be bipartite by including the KR residues 13 amino acids upstream from the NLS sequence ($KR_{X13}RRRK$), at positions 279-280. The activity of the NLS is not controlled by phosphorylation of phosphorylatable amino acids in its vicinity (Y292 and T300), as revealed by non-phosphorylatable (Y292A and T300A) and phosphomimetic (Y292D and T300D, and later Y292E and T300E) amino acid substitutions.

To see, whether the NLS identified by us in Drosophila moesin can also be found in the ERM proteins of other species, we performed multiple sequence alignments of 24 ERM proteins found in 18 different species. The analysis revealed, that the bipartite NLS identified in moesin is evolutionary highly conserved. This conservation not only applies to the two parts of the NLS motif itself, but also to the distance between them. The high degree of evolutionary conservation of the NLS and the immediate region surrounding it indicates that the residues found here are indeed important for the proper functioning of ERM proteins.

Closed conformation is preferred for nuclear import

ERM proteins exist in open (active) or closed (inactive) conformational states in the cell. The open conformation is stabilized by phosphorylation of the threonine at position 559. In order to investigate, which conformational form of the protein might be import-

competent, we generated the point mutants MoeT559D (threonine 559 mutated to aspartic acid) and MoeT559A (threonine 599 mutated to alanine), which were already described in the literature previously (Polosello et al., 2002). First we tested whether by cortical FRAP experiments whether there is a real difference between the activity of the two mutant proteins. These experiments confirmed that the two mutant forms behave as expected. The active mutant, MoeT559D preferentially localized to the cell cortex and exhibited a lower turnover rate after photobleaching, indicating that it forms strong molecular interactions near the plasma membrane. In contrast, the inactive mutant, MoeT559A showed a nearly homogeneous distribution in the cytoplasm and its turnover rate was greater in the cortical FRAPs, meaning that it does not take part in strong protein-protein interactions there.

Next, we carried out nuclear import FRAP assays with the MoeT559 mutants. The resulting FRAP curves revealed, that the nuclear import dynamics of the two mutant proteins are very similar. Both mutants exhibit a recovery curve similar to wild type moesin, indicating a slow and steady influx of the mutant forms into the nucleus. This suggests, that both mutants are able to enter the nucleus similarly to the wild type protein and that the dynamics of moesin's nuclear import is not regulated through its conformation.

The results we obtained using the nuclear FRAP assay were further corroborated by immunostaining of cells expressing the GFP-tagged mutants, MoeT559D and MoeT559A. Upon induction of the nuclear import of moesin by *Rae1* RNAi, both mutant forms were able to enter and accumulate in the nucleus, just like the wild type of the protein. However, the quantification of the data showed, that the active mutant form is present in the nucleus in significantly smaller quantities under both uninduced and induced conditions, suggesting a preference for the closed conformation of the protein for nuclear import.

To confirm or disprove the idea that the closed conformation is preferred for nuclear entry, we examined how the inhibition of the Slik kinase, which phosphorylates T559 in Drosophila moesin, affects the import. *Slik* RNAi treatment alone increased the amount of moesin in the nucleus even without the induction of import by *Rae1* RNAi. This result provides more evidence that the non-phosphorylated form of moesin is favored in nuclear import.

F-actin binding does not regulate the nuclear import of moesin

In order to investigate, whether actin has a role in the regulation of the subcellular localization of moesin, we treated Drosophila S2R+ cells expressing Moe-GFP with Jasplakinolide, a cytoskeletal drug that promotes actin polymerization and thereby decreases the amount of monomeric actin in the cytoplasm. *Rae1* RNAi was also applied to induce the nuclear import of moesin. We found, that increasing the amount of F-actin and simultaneously reducing the pool of available G-actin does not result in any noticeable change in the nuclear import of moesin, the protein can still accumulate in the nucleus upon import induction. This indicates that monomeric actin is neither hindering nor is necessary for the nuclear translocation of moesin.

In a follow up experiment, the amount of free monomeric actin was increased with the help of another cytoskeletal drug. The sponge toxin Latrunculin A prevents actin polymerization and enhances actin depolymerization, resulting in the increase of the amount of free G-actin in the cell. Based on the results we concluded that the depolymerization of

the actin network had no effect on the nuclear import of the protein, which means also that it is not F-actin binding that inhibits the nuclear import of activated moesin.

To further confirm, that F-actin binding is not preventing the nuclear import of moesin, the C-terminal deletion mutant, Moe-ΔC was created. The C-terminal domain is responsible for F-actin binding, therefore Moe-ΔC is incapable of binding to filamentous actin. This truncated protein form was unable to enter the nucleus upon induction of import, providing further evidence that it is not the F-actin binding that retains moesin in the cytoplasm.

Moesin has a functional cytoplasmic retention signal

The FERM domain containing protein, merlin is a close relative of ERM proteins. The second exon of the *merlin* gene encodes a short sequence of its FERM domain, which has been described to be responsible for the cytoplasmic retention of the protein. Upon deletion of this exon, the mutant protein is able to enter the nucleus in a considerable amount. We compared exon 2 of human merlin to the corresponding region of Drosophila moesin, and found a high degree of similarity with multiple identical amino acids.

We decided to test whether moesin also possesses a retention motif in this region (hereafter referred to as: Cytoplasmic Retention Signal or CRS for short), that could be responsible for its retention in the cytoplasm. To this aim, we generated a deletion mutant called MoeΔCRS, in which 10 amino acids were deleted from the middle of the potential, 25 amino acids-long CRS sequence of moesin. We found that the subcellular localization of the GFP-tagged MoeΔCRS mutant protein is remarkably different from that of wild type moesin. Interestingly, MoeΔCRS localizes primarily to the plasma membrane and also to filopodia. However, the amount of the mutant protein in the nucleus is not markedly different from that of the wild type.

In order to confirm the possible role the CRS might play in the regulation of the nuclear import of moesin, we carried out nuclear import FRAP assays on S2R+ cells expressing GFP-tagged MoeΔCRS. We found that the dynamics of import changed dramatically compared to either the wild type, or any other mutant protein form we have created and tested so far. The FRAP curve showed, that the MoeΔCRS mutant is imported into the nucleus very dynamically.

A sequence of 25 amino acids is responsible for the cytoplasmic retention of Drosophila moesin

To further study and characterize the functionality of this newly identified CRS sequence, we designed an experimental system in which a control protein, the GFP, that freely moves between the nucleus and the cytoplasm was tagged with different parts of the moesin CRS and its surroundings. Three GFP constructs were designed and created (GFP-CoreCRS, GFP-CRS, GFP-Exon10) along with two control constructs (GFP and GFP-R60). We found that the 10 amino acids of the CoreCRS tag were unable to inhibit the nuclear entry of GFP, while the CRS (25 amino acids) and Exon10 (60 amino acids containing the full CRS) tags clearly inhibited the nuclear localization of GFP. These results tell us, that the 25 amino acids-long CRS sequence is sufficient to prevent nuclear entry of

a protein and is most likely responsible for this phenomenon in the case of whole-length, wild type moesin.

To further confirm our finding that the CRS sequence of moesin in indeed functional, we analyzed the nuclear import dynamics of some of the constructs used in the previous experiment. The controls, GFP and GFP-R60 exhibited a highly dynamic import FRAP curve, indicating free and unrestricted diffusion into the nucleus. In contrast, GFP-Exon10, which contains the CRS sequence, shows a linear FRAP curve with small inclination, which is almost identical to that of wild type moesin.

The CRS identified in moesin is evolutionarily conserved

To analyze the evolutionary conservation of the CRS, we aligned the sequences of 21 FERM domain-containing proteins found in 12 species. The alignment revealed that a highly conserved region of 22 amino acids can be identified in the CRS region of all proteins examined. Out of the 22 amino acids, five are the same in all sequences analyzed, while 8 other positions are filled by amino acids with similar chemical properties and thus, they contribute to the high degree of conservation. This short stretch of 22 amino acids identified by the alignment shows a high degree of conservation across multiple species, indicating that the CRS might be indeed an important part of the FERM domain by playing a role in the regulation of the subcellular localization of FERM domain-containing proteins.

RESULTS AND CONCLUSIONS

Using the nuclear import FRAP technique, we proved that the nuclear import of the Drosophila moesin protein is an active, regulated process. To enter the nucleus, moesin utilizes a bipartite NLS sequence, which was previously identified in our lab. With the help of multiple alignment and comparison of sequences of the NLS region from different species we showed that the NLS identified in moesin has a high degree of evolutionary conservation.

Even though we know that moesin has a functional NLS, under normal conditions the protein localizes mainly to the cytoplasm, suggesting that the activity of the NLS is downregulated. One possible regulatory mechanism is, that the import activity of the protein is controlled by its conformation. We found that both open and closed forms of moesin can enter the nucleus, however the inactive, closed form seems to be preferred for nuclear import.

Next we investigated whether F-actin binding plays a role in the regulation of the nuclear import of moesin, and we concluded that the ratio of the amount of filamentous and monomeric actin in the cell had no significant effect on the subcellular localization of moesin. This result was further supported by the fact that the C-terminally truncated mutant form of moesin, Moe-ΔC, was unable to enter the nucleus and accumulate there.

In another FERM domain containing protein, merlin, a region was identified being responsible for the cytoplasmic retention of the protein. When we compared this region with the corresponding amino acids of moesin, we found a high degree of similarity. We tested the functionality of this region by multiple methods. The results of these experiments all supported the notion, that moesin has a functional cytoplasmic retention sequence. We also looked at the conservation of this CRS motif and found a high degree of similarity between aligned sequences, indicating evolutionary conservation of the CRS.

Based on the findings presented in this dissertation, we can create a model for the regulation of nuclear import of moesin. According to this, under uninduced conditions, moesin is mainly localized in the cytoplasm of interphase cells, because the nuclear import of the protein is strongly hindered. This suppression is achieved by multiple mechanisms working in cooperation. However, in certain cases, such as for example increased transcriptional activity of the cell, moesin enters the nucleus, where its amount increases to relatively high levels. This increased nuclear import activity is most likely caused by the stress-induced release of moesin from the binding of the CRS-recognizing cytoplasmic factor, thereby making the NLS of moesin accessible for its importin, which finally transports moesin into the nucleus via the NPC.

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