Identification of a novel function played by Importin-α2 protein during *Drosophila* oogenesis

Ph.D. thesis

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Szeged, 2003

Introduction

In eucaryotic cells the macromolecular transport between the nucleus and the cytoplasm is a selective signal- and receptor-mediated process. In the cytoplasm, the Importin-α adapter protein recognizes the nuclear localization signal (NLS) peptide of the cargo proteins and in a heterotrimeric complex with Importin-β they translocate into the nucleus. The high nuclear concentration of the Ran-GTP dissociates this complex and then the transport receptors are recycled. However, it was recently found that the Importin proteins and Ran exert other functions as well, not related with nucleocytoplasmic transport. *In vitro* studies show that Ran-GTP elicits spontaneous microtubule polymerization and spindle assembly in cell-free *Xenopus* egg extracts. Recently, the effect of Ran was found to be mediated by Importin-α and –β, which can bind to and inhibit the function of the microtubule-assotiated TPX2 and NuMA proteins. TPX2 and NuMA become released from the Importins by Ran-GTP and can thus promote spindle and centriole formation. Similar interaction may explain the involvement of Importins and Ran in nuclear envelope assembly following mitosis. In this way, Importin-α, Importin-β and Ran participates in three distinct processes mechanisticaly in the same way.

We have examined the function of *Drosophila* Importin-α2 protein during oogenesis and spermatogenesis. We have found that during spermatogenesis Importin-α2 showed a cell cycle-dependent nuclear accumulation that was similar to the previously described distribution during early embryogenesis. This may indicate that during both spermatogenesis and embryogenesis Importin-α2 participates in the

nuclear import of cell cycle-specific molecules. However, during oogenesis Importin-α2 remains cytoplasmic in steady-state, in contrast to the nuclear accumulation of other Importins, suggesting a possible cytoplasmic function. Here I report on the critical role played by Importin-α2 during *Drosophila* egg chamber development and describe a novel, entirely cytoplasmic function distinct from the nuclear transport.

A characteristic of gametogenesis in *Drosophila* is that sperm and oocyte develop within a cyst of cells. These are derived from a cystoblast that undergoes four rounds of mitosis with incomplete cytokinesis to produce 16 cells interconnected through 15 cytoplasmic bridges called ring canals. The ring canals are built around the remnants of the cleavage furrow and contain several of its molecular components such as F-actin, Anillin, Filamin or Kelch. In oogenesis, one cell differentiates as oocyte and the remaining 15 cells become the highly polyploid nurse cells that are synthetically active and contribute cytoplasm to the oocyte. The process of this transfer can be divided into two phase. The first phase that take place up to stage 10 of egg chamber development, is characterized by a slow and selective transfer of RNA transcripts and proteins, resulting more particular in the deposition of embryonic determinants. Then, from stage 10 a second non-selective phase of transfer results in a rapid discharge (dumping) of the cytoplasmic content of the nurse cells into the oocyte, leaving behind the nuclei in the apoptotic nurse cell remnants.

Methods

> Genetic work with different *Drosophila melanogaster* stocks (crossings, rescue with transgenes, etc.)

> P element-mediated germ cell transformation

> Immunocytochemistry of ovaries and embryos

> β-galactosidase activity staining of ovaries

> Nucleic acid procedures: *in vitro* site-directed mutagenesis, Myc- and ProteinA-tagging of wild type and mutant *importin-α2* genes, PCR, cloning, sequencing, etc.

> Protein procedures: *in vitro* translation, co-immunoprecipitation, Importin-α2 complex purification, microfilament association assay, Western blot

> Microscopy: work with fluorescence microscope, confocal laser scanning microscope, differential-polarization laser scanning microscope and scanning electron microscope

Results and Discussion

We have established an intrinsic deletion in the *Drosophila importin-α2* (*imp-α2*^{D14}) gene and have found that it causes partial male and full female sterility. Examination of *imp-α2*^{D14} ovaries showed that egg development was retarded producing smaller eggs than the normal. This phenotype is a characteristic of dumpless mutations in which the transfer of nurse cells cytoplasm into the oocyte is incomplete. In the known dumpless mutants, the failure in this transport may be explained by three distinct defects in which in each case the actin cytoskeleton is

involved. This promed us to investigate the distribution of F-actin in wild type and mutant *imp*-$\alpha 2^{D14}$ egg chambers. Phenotypic analysis reveals, that the block of this transport in *imp*-$\alpha 2^{D14}$ egg chambers is result of the partially occluded ring canals. In *imp*-$\alpha 2^{D14}$ mutant ring canals the actin filaments and all of the proteins bound to the actin (Filamin, Hts-RC, Pav-KLP, PY-epitopes, *etc*.) extend into the lumen of the ring canals, whereas wild type ring canals formed a compact ring. These data indicate that the Importin-α2 function is involved in the assembly of functional ring canals. Despite it, we were unable to detect any trace of this protein in association with wild type ring canals indicating, that Importin-α2 may affect the ring canal assembly indirectly, without beeing a component of it. A very similar mutant ring canal structure could be observed in *kelch*^{DE1} null mutant egg chambers. However, Kelch protein is a stable constituent of the wild type ring canals and is responsible for the dynamic chross-linking of the ring canals' actin filaments. To determine whether Kelch or any other known ring canal components involved in ring canal assembly were missing from *imp*-$\alpha 2^{D14}$ mutant ring canals, we performed the immunostaining of these proteins in wild type, *imp*-$\alpha 2^{D14}$ and *kelch*^{DE1} mutant egg chambers. These examinations revealed that no Kelch protein could be detected in neither *imp*-$\alpha 2^{D14}$ nor *kelch*^{DE1} mutant ring canals, although Western blot analysis showed that Kelch protein was normally expressed in *imp*-$\alpha 2^{D14}$ egg chambers. We can thus infer that the organisation of actin filments and associated proteins in the ring canals depends on Kelch assotiation with these structures. These data show, that Kelch is a downstream target of Importin-α2 and that Importin-α2 plays a role in the proper targeting of Kelch to the ring canals.

To elucidate this novel function of Importin-α2 and to determine the domains indispensable for ring canal assembly, we performed an *in vitro* site-directed mutagenesis of the *importin-α2* gene. Mainly based on the crystallographic analysis made on yeast Importin-α protein, we targeted the Importin-β binding domain (IBB), NLS-binding domain, CAS-binding domain and some putative phosphorylation residues of Importin-α2 protein. The mutant constructs were transformed into the flies and their effect was investigated on *imp-α2*^{D14} null mutant background. We found that the IBB domain and the residues necessary for NLS-binding were also important for the *Drosophila* oogenesis and for the deposition of Kelch protein to the ring canal. This observation suggess that Importin-α2 protein may function in ring canal assembly mechanistically in the same way as the Importin-α proteins participate in the nucleocytoplasmic transport, microtubule assambly or nuclear envelope assembly, mentioned above.

To get a better idea on how Importin-α2 protein participates in ring canal assembly, we performed a complex purification by overexpressing ProteinA-tagged wild type and mutant Importin-α2 proteins in *Drosophila* ovaries. Among the numerous isolated proteins we found that Importin-α2 formed a complex with the Kelch protein. However, the mutant forms of Importin-α2 proteins, in which the IBB domain and the NLS-binding residues were targeted, could not make the complex with Kelch protein. These observations strongly indicate, that the interaction between Importin-α2 and Kelch depends on NLS binding. Since Kelch protein has no NLS sequence (only a stretch of basic residues), we can envisage that Importin-α2 transports Kelch to the ring canals through an intermediary of an unknown component.

The *in vitro* generated mutant, where the CAS-binding residues (responsible for the recyclization of Importin-α protein from nucleus) were targeted, was trapped into the nuclei and completely depleted from the cytoplasm of each cells of egg chambers. This observation unequivocally verify that during oogenesis Importin-α2 protein takes part in nucleocytoplasmic transport. However, the intact nuclear import of NLS-reporter proteins (NLS-GFP, NLS-βGal, Pav-KLP, *etc*.) on *imp-α2^{D14}* mutant background, moreover the subcellular distribution of Importin-α2 suggest that this protein is not responsible for the bulk nucler transport during oogenesis, but may be involved in nuclear import of some specific factors. Another characteristic of Importin-α2 cytoplasmic localisation is its cortical colocalisation with F-actin. To characterise their relationship we depolymerised the cortical actin filaments of egg chambers and found that it caused a dramatic redistribution of Importin-α2 protein. Importin-α2 accumulated in the nuclei of nurse cells and oocyte, suggesting that F-actin may regulate the nuclear import of this protein. Furthermore, we found that the F-actin binding is dependent on NLS-binding and that it is common in the case of other Importins. This indicates that F-actin network may serve as a major organisator of the nuclear transport during *Drosophila* oogenesis.

It was previously describe, that the Importin-α proteins contain two NLS-binding sites: a major and a small one. In the absence of Importin-β the major NLS-binding site serve as an auto-inhibition domain, too. The endogenous NLS sequence of Importin-α proteins is bound by this major NLS-binding site preventing the interaction with other NLS sequences.

Since the interaction between Importin-α2 and Kelch depends on NLS squence, we made a further dissection of the NLS-binding domains of Importin-α2 protein. When only the small NLS-binding domain was

targeted, the Importin-α2 did not lost its function during oogenesis. However, when only the major NLS-binding site was modified, the Importin-α2 lost its function. This suggests that for the function of Importin-α2 during oogenesis only the major NLS-binding site is critical. Additionlally, this mutant had a very strong dominant negative effect. Overexpressing in germ cells or in other proliferating tissues it caused cell lethality. We belive that a possible explanation for it is that this mutant protein lost its ability for the auto-inhibition and that through its still functional small NLS-binding domain it may irreversibly bind and prevent the function of some other proteins. To prove it we are planning some further biochemical experiments.

In my Ph.D. thesis I describe a novel function of Importin-α2 played during *Drosophila* oogenesis. I presented that in this function Importin-α2 uses the same domains and mechanistically function in a similar manner as the Importin proteins function in the previously described functions. By a set of *in vitro* mutants of *importin-α2* gene I made a detailed description of the function of Importin-α2 protein and found the first dominant negatve allel of this gene.

List of publications

1. Tibor Török, **Mátyás Gorjánácz**, Peter J. Bryant and István Kiss (2000). Prod is a novel DNA-binding protein that binds to the 1,686 g/cm^3 bp satellite repeat of *Drosophila melanogaster*. ***Nucleic Acids Research*** **28**: 3551-3557.

2. **Mátyás Gorjánácz**, Géza Ádám, István Török, Bernard M. Mechler, Szlanka Tamás and István Kiss (2002). Importin-α2 is critically required for the assembly of ring canals during *Drosophila* oogenesis. ***Developmental Biology*** **251**: 271-282.

3. Marianna Giarrè, István Török, Rolf Schmitt, **Mátyás Gorjánácz**, István Kiss and Bernard M. Mechler (2002). Patterns of importin-α expression during *Drosophila* spermatogenesis. ***Journal of Structural Biology*** **140**: 279-290.