Ph.D. thesis

The catalytic module of the anaphase promoting complex in *Drosophila melanogaster*

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1. Introduction

Crucial cell cycle events, such as chromosome separation and exit from mitosis are regulated by the ubiquitin-dependent protein degradation system. This process consists of two distinct steps. First, target proteins are marked with polyubiquitin chains by an enzyme cascade, and then the marked proteins are recognized, and degraded by the 26S proteasome. The first step in this process is the activation of the ubiquitin molecule carried out by the E1 ubiquitin-activating enzyme. During this process, a thiol ester bond forms between the carboxy-terminal glycine of ubiquitin and the active-site cystein of the E1 ubiquitin-activating enzyme. Following that, the activated ubiquitin is transferred to the E2 ubiquitin-conjugating enzyme. Finally, the activated ubiquitin is coupled to a lysine residue of the targeted protein. This process needs another enzyme activity, the E3 ubiquitin-protein ligase. The key component of this proteolytic system is the E3 ubiquitin-protein ligase, which provides both platform and substrate specificity for the ubiquitylation process.

The mitosis and G1 phases of the cell cycle are regulated by the APC/C (anaphase promoting complex) ubiquitin-protein ligase. The APC/C mediates the degradation of securin and mitotic cyclins that induce sister chromatids separation and exit from mitosis.

The APC/C is a large, 1.5 MDa protein complex, that contains at least 12 or 13 subunits. The sequence and topology of these subunits appear to be evolutionary conserved. Though we know the general function and its basic regulatory mechanism, there are still many unanswered questions about the structure and role of the APC/C.

2. Specific aims

The aim of our work is to understand the function and structure of the APC/C in the fruit fly model organism. In order to understand the role of APC/C subunits and to get insight into their structure and function, we generated knock out mutants on genes encoding different subunits of APC/C and we made detailed analysis of their phenotypes. My research plan was to analyze the Apc11 subunit, which compose the catalytic subcomplex of the APC/C throughout these steps:

1. Genetic, cell biological and cytological characterization of the hypomorph alleles of Apc11.
2. Isolation Apc11 null mutant using P element remobilization and its characterization.
3. To determine the functional orthologue of the Apc11 in Drosophila by heterologous complementation experiments.
4. Analysis of protein interaction between the Apc11 and APC/C subunits using yeast two-hybrid assay and genetic interaction experiments.
5. To determine the interaction between the APC/C and E2 enzymes using yeast three-hybrid assay and genetic interaction experiments.

3. Methods

1. Recombinant DNA technology
2. Semi-quantitative reverse transcription coupled PCR (RT-PCR)
3. RACE reaction

4. Western blot
5. Yeast two- and three-hybrid assay
6. Determining β-galactozidase activity
7. Heterologous complementation experiments
8. P element remobilization
9. Neuroblast preparation and cytological characterization
10. Drosophila feeding experiments

4. Results

1. Our work was started with the identification of the *l(2)03424* P element insertion mutant, which showed pharate adult lethality and had reduced eyes, wings and bristle defects. Imaginal discs stained with acridine orange showed elevated level of apoptosis, therefore we renamed the affected gene as *lemming*. In addition to this, we have isolated and characterized non-complementing hypomorph ($lmg^{EY11317}$ and lmg^{J023}) and null (lmg^{138}) alleles that together form an allelic series. The common feature of the different alleles is their late lethality. Remobilization of the P element in the insertion lines ($lmg^{EY11317}$ and lmg^{J023}) frequently caused reversion of the mutant phenotype to wild type, suggesting that these insertions were indeed directly responsible for the mutant phenotype.

2. Cytological analysis of orcein stained larval neuroblast preparations revealed obvious mitotic abnormalities in all mutant alleles. The proportions of cells in mitosis were much higher (mitotic index) in mutant preparations than in wild types, and most of these cells were in prometa- or metaphase-like stage. Cells in prometaphase or metaphase had highly

condensed chromosomes, and cell in anaphase were rare but most of them had abnormal chromosomes. In addition to this, polyploid cells were frequently observed in mutant preparations, though such cells could never be seen in wild type preparations. Most of the polyploid cells had overcondensed chromosomes. All of the mitotic phenotypes listed above suggest that in *lmg* mutants the cell cycle is delayed or arrested in prometa- or metaphase-like stage.

3. The *lmg* gene was cloned by plasmid rescue of the genomic DNA flanking the P element insertions. This was followed by sequence analyses of plasmid rescue fragments, and comparative analyses of genomic and cDNA sequences were used to determine the structure of the *lmg* locus. It turned out that the *lmg* gene is intronless and codes for a ~2 kb mRNA which contains two open reading frames (ORF), thus it is dicistronic. The 5' ORF was designated as *lmgA*, and the 3' ORF as *lmgB*. The *lmgA* encodes a small, putative polypeptide of 85 amino acids (~ 10 kDa), which shows more than 80% sequence similarity with the APC11 subunit of the human APC/C, and contains a RING finger motif characteristic to known APC11 subunits. The expression of *lmgA* alone was sufficient to fully rescue the lethal, morphological and mitotic phenotypes of lmg^{138} null mutant. The *lmgB* ORF codes for a putative polypeptide of 365 amino acids, with apparently no known functional domains. Although the LmgB protein sequence is conserved in Drosophilidae, homologous sequences could not be found in other species.

4. We have analyzed the expression pattern of *lmg* locus in different developmental stages of Drosophila. According to our results, the dicistronic message could be the main product of *lmg* transcription,

however it could not be excluded that monocistronic mRNAs might be produced. If monocistronic messages were present, their ratio and expression profile, did not differ significantly from that of the dicistronic message. Beyond that, we could not detect efficient translation of LmgB in S2 cells in such circumstances where the transcription of the *lmgA* and the presence of LmgA could be detected.

5. The well-known substrates of the APC/C, the mitotic cyclins are degraded before or during the metaphase-anaphase transition, thereby permitting mitotic exit. Loss of the essential APC/C subunits causes stabilization and accumulation of the mitotic cyclins within cells. Our immunostaining experiments in *lmg* mutant neuroblasts showed that cells arrested in mitosis accumulate both Cyclin A and Cyclin B. These results show that the Lmg protein has a role in of mitotic cyclin degradation.

6. The high sequence similarity to the human Apc11, and the phenotypes mentioned above suggest that the *lmgA* codes for this evolutionary conserved subunit in Drosophila. This is further supported by our heterologous complementation experiments, in which *lmgA* was able to complement the thermosensitive lethal phenotype of budding yeast (*Saccharomyces cerevisiae*) Apc11-deficient cells. Accordingly, the LmgA protein in fruit flies is the functional orthologue of the Apc11 subunit.

7. It is known that in yeast and human cells the Apc2 subunit binds to Apc11, and they together form the catalytic sub-complex of the APC/C. Likewise, some data suggest the E2 ubiquitin-conjugating enzymes interact with either the Apc2 or the Apc11 subunits. We have demonstrated in yeast two-hybrid experiments, that the Drosophila LmgA/Apc11 subunit interacted

with the Apc2/Mr subunit. Surprisingly, in similar experiments we could not detect any interaction between the Vihar E2 enzyme and either LmgA/Apc11 or Apc2/Mr subunits. However, we could observe interaction if all three proteins were present in the cells. This result suggests that LmgA/Apc11 and the Apc2/Mr together form a platform for Vihar binding, so they constitute a ternary complex.

8. We have also demonstrated that similarly to Vihar, the LmgA/Apc11 and Apc2/Mr subunits form together a binding site for the product of the *CG8188* gene; therefore it is possible that this protein and Vihar compete for the same binding site. This protein shows more than 60% similarity to the human Ube2S E2 ubiquitin-conjugating enzyme, and considered to be the homologous enzyme in Drosophila (DmUbe2S). It was shown recently that the lysine K11-linked ubiquitin chains are assembled by two E2 enzymes in human cells. Ube2S elongates ubiquitin chains after the substrates become pre-ubiqitinated by UbcH10 or UbcH5-type E2 enzymes. We found out that hypomorph and null alleles of the *DmUbe2S* gene are viable, and do not show any mitotic phenotypes, suggesting that the DmUbe2S enzyme either has no E2 activity or it is negligible during normal development. However, the *DmUbe2S* mutants showed hypersensitivity to chemicals activating the spindle-assembly checkpoint, such as colchicine or docetaxel and this phenotype could be rescued in *mad2* mutants with inactivated spindle-assembly checkpoint. This feature of the *DmUbe2S* mutants is similar to the behavior of human cells lacking Ube2S function and supports the idea that Ube2S is important for silencing the spindle-assembly checkpoint.

5. Summary

1. The *lemming* locus codes for a dicistronic gene in Drosophila.
2. It's first ORF, the *lmgA* codes for the Apc11 subunit of the APC/C ubiquitin-ligase.
3. The genetic interactions of *lmgA* with the *apc2/mr, vihar* and *Dmube2S* genes and the physical interaction of their gene products – LmgA-Apc2-Vihar/Dmube2S – suggest that this ternary complex represents the same catalytic sub-complex of the APC/C, as the one identified by biochemical means in yeast and mammalian cells.
4. We have identified the Drosophila orthologue of the gene coding for the human Ube2S ubiquitin-conjugating enzyme.
5. It was shown that the APC/C-dependent E2 activity of DmUbe2S is dispensable in this organism, but its role appears to be critical for the normal functioning of the spindle-assembly checkpoint.

6. Publications

Nagy O, Pal M, Udvardy A, Shirras C., Boros I., Shirras A., Deak P.
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