Genetic and molecular analysis of ecdyson receptor isoforms in *Drosophila*

Summary of Ph.D. thesis

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INTRODUCTION

The molting hormone ecdysone is a key regulator of insect development. Pulses of ecdysone appearing in certain developmental stages direct the ecdysone-dependent developmental steps. The ecdysone manifests its effects via interaction with the ecdysone receptor, which is a transcription factor, activated by hormone-binding. To our knowledge, ecdysone acts by the same mechanism in different developmental stages and target tissues. Increase of ecdysone concentration induces a gene cascade. A small number of primary-response early genes encode transcription factors that coordinate the induction of a large set of secondary-response, so called late genes, which mediate stage- and tissue-specific biological responses. In *Drosophila* the ecdysone receptor is a heterodimer of two nuclear receptor proteins, EcR and USP encoded by the genes *EcR* (*ecdysone receptor*) and *usp* (*ultrspiracle*) respectively (Koelle et al, 1991; Yao et al., 1992; Thomas et al., 1993). Since the *EcR* gene is itself ecdysone-induced (Karim et al., 1992; Deák P et al., 1988), the hormone-sensitivity of target tissues occur only in specific developmental stages marked by high ecdysone concentrations.

The structure of the *Drosophila EcR* gene is complex. It contains two separated transcription start sites and codes for three protein isoforms (EcRA, EcRB1, EcRB2) produced by alternative splicing (Talbot et al., 1993). The exact role of different EcR isoforms is unknown. Any EcR isoform can compensate the failure of another one if large amount of it is expressed ectopically (Bender et al., 1997), however it is not clear to what extent this replacement works under normal conditions. On the other hand, there are data for the specific function of particular *EcR* isoforms, as well (Talbot et al, 1993; Mouillet et al., 2001). Mutant alleles which inactivate only the *EcRB1* isoform, or the *EcRB1* and *EcRB2*, or all three *EcR* izoforms together are described in the literature (Bender et al., 1997; Schubiger et al., 1998). However, the lack of mutations affecting only *EcRA* or the *EcRB2* isoform does not allow the direct functional study of these two *EcR* isoforms.

Therefore our goal was to shed light on the role of the *EcR* isoforms by identification and characterisation of further *EcR* mutant alleles, and determine if any additional, so far unidentified isoform(s) exists.

OBJECTIVES

1. Identification of new *EcR* isoforms
2. Analyses of the expression of *EcR* isoforms by RT-PCR
3. Identification of P-insertion lethal *EcR* alleles
4. Phenotypic analysis of the lethal P-insertion *EcR* mutants
5. Rescuing the lethal phenotypes of *EcR* mutants using transgenes of *EcR* isoforms
6. Identification mutant alleles which inactivate the EcRA protein synthesis
7. Analysis of the phenotype results from the lack of EcRA protein

METHODS

Classical genetic methods

- Fly stocks and crosses
- Complementation tests for identification of lethal P-insertion alleles
- Determination of lethal phase
- Analysis of fertility

Molecular methods

- Plasmid rescue
- Southern hybridisation
- DNa sequencing
- PCR
- RT-PCR
- Western blot

Cytological methods

- Salivary gland polytene chromosome *in situ* hybridisation
- Microscopic analysis of larval tissues
- DAPI staining of larval tissues

RESULTS AND DISCUSSIONS

1. As a first step, we screened the collection of Berkeley *Drosophila* Genome Project (BDGP) EST sequences and identified a new *EcR* isoform, *EcRC*. The transcription start site of *EcRC* differs from that of any other *EcR* isoforms, but it encodes a protein identical with EcRA. The different transcription start sites of *EcRA* and *EcRC* isoforms suggest that mRNAs giving rise to EcRA protein can be produced from different promoters. The exact role of this complex regulation of EcRA production is not clear, yet. It is possible, that the large amount of EcRA protein, required at certain developmental stages, could be produced on this way. Alternatively, it is also possible that the fine temporal and spatial tuning of EcRA expression plays an important role and this requires two isoforms with altering regulation.

2. We studied the expression of the *EcR* isoforms by RT-PCR during *Drosophila* development. We found the expression pattern of the *EcRB1* and the *EcRB2* isoforms identical, as expected by their common transcription start site. The *EcRC* and the *EcRA* isoforms showed similar expression pattern. *EcRC* and *EcRA* mRNAs could also be detected in all developmental stages studied. We observed differences in the expression pattern of the *EcRA* and *EcRC*. Namely, in the early embryos and in adult ovaries the *EcRC* mRNA was more abundant.

3. In order to identify further *EcR* alleles we screened a recessive lethal P-insertional mutant collection and found nine independent *EcR* mutant lines. We determined the positions of the P-insertions and the lethal phenotype of the alleles. Furthermore we analysed which *EcR* izoforms was affected in the mutants. The *P+12319* allele inactivates all *EcR* isoforms. The *P-9* results in a reduced expression of *EcRB* isoforms while it does not change the *EcRA* and *EcRC*. The remaining seven lesions (*P-25*, *P-40*, *P-191*^{27}, *P-191*^{77}, *P-191*^{101}, *P-1391*, *P*-5738) are amorph alleles of *EcRA* and *EcRC* and hypomorph alleles of *EcRB* isoforms. The *P+12319* allele is embrionic lethal, whereas all of the other lethal P-insertional mutants execute normal embrionic and larval development. Thus, we can conclude that EcRA protein is not essential in these developmental stages.

4. In order to identify mutations which make possible direct study of the role of *EcRA* and *EcRC* isoforms, from databases we chose two P-insertional mutant lines (*l(2)06410* and *EP(2)2509*) which are known to have insertion positions in introns of *EcRA* and *EcRC*. Further analysis of these mutants revealed inhibition of EcRA protein synthesis. The *l(2)06410* is amorph and the *EP(2)2509* is strong hypomorph allele of both *EcRA* and *EcRC* isoforms. The *l(2)06410* insertion was originally isolated as a male-sterile, semilethal allele of *the long island expressway* (*lie*) gene (Castrillon et al., 1993). Complementation tests, using deficiencies which remove the region around the position of insertion, showed that the male-sterile, semilethal phenotype is independent from the *l(2)06410* insertion. The *l(2)06410* and *EP(2)2509* mutations does not influence viability and morphology of the flies. Consequently, the EcRA protein is not essential at any stage of the *Drorsophila* development. The lack of EcRA protein did not cause any defect in the male fertility, but the oogenesis of *l(2)06410/EcR*^{554fs} and *EP(2)2509/EcR*^{554fs} females is slightly affected. This phenotype might be due to lack of EcRA and simultaneously reduced level of EcRB proteins, since the amorph *EcR*^{554fs} allele inactivates all *EcR* isoforms, and the *l(2)06410/EP(2)2509* heterozygotes do not show any abnormality in oogenesis.

Although EcRA is present in all developmental stages, and is produced by two independently regulated transcription units, its lack does not result in a distinguishable phenotype. From the observation that EcRA is not essential but its effect is seen if the other EcR isoforms are deleted, one might speculate whether EcRA serves as a back up to ensure EcR function or it has specific role which is important for the free-living flies but can not be studied under laboratory conditions.

5. Phenotypical comparison of *EcR* alleles described by us and others indicated that the different EcR isoforms could mutually replace each other.The null mutants eliminating all isoforms are embrionic lethal (Bender et al., 1997), however either EcRA alone (Schubiger et al., 1998) or the two EcRB proteins (*l(2)06410, EP(2)2509*) are sufficient for normal embrionic development. These observations demonstrate the overlapping and redundant function of the EcR isoforms in the embrionic stage.

The *EcRA* null mutant larvae execute normal development (*l(2)06410, EP(2)2509*), even in combination with reduced levels of EcRB proteins (*P-25, P-40, P-191*^{27}, *P-1391*, *P*-5738). Complete arrest of larval development occurs in null mutants which inactivate all *EcR* isoforms (Li et al., 2000), whereas the lack of both *EcRB* isoforms causes a

weaker phenotype, allowing development some of the mutant animals (Schubiger et al., 1998). The failure of EcRB1 has a moderate effect, 40-60% of the mutant larvae can molt and survive until L3 stage (Bender et al, 1997).These phenotypes show that the different *EcR* isoforms have not specific role in larval development. On the other hand, all isoforms participate in the regulation of larval moltings. The observation, that null alleles which eliminate all *EcR* isoforms have stronger phenotype than the EcRB1-B2 double mutants, demonstrates that the redundant EcRA protein participates in the larval development as well. EcRB1 and EcRB2 proteins together are sufficient for proper development, whereas EcRA or EcRA-EcRB2 combinations are not.

EcRA is not needed for metamorphosis (*l(2)06410, EP(2)2509*), but the mutants of the other known *EcR* alleles are arrested during metamorphosis. The phenotypes of mutants do not support the theory that the different EcR proteins have specific function, and that the fate of tissues during metamorphosis are determined by the predominant EcR isoform expressed in the given tissue (Talbot et al., 1993). Support this notion that the development of imaginal discs which contain a large amount of EcRA, and the development of midgut which contains dominantly EcRB1, is arrested similarly, earlier in those mutants which are *EcRA-C* null and strong *EcRB* hypomorph (*P-25, P-40*) and later in those which are *EcRA-C* null and weak *EcRB* hypomorph (*P-191*^{27}, *P-1391, P*-5738). Furthermore, perfect adult body parts develop from imaginal discs in the lack of EcRA (*l(2)06410, EP(2)2509*).

6. A further arguments for the observation that the *EcR* isoforms can replace each other in the metamorphosis is that the lethal phenotype of the *EcRA-C* null and *EcRB* hypomorph P-element insertion mutants can be rescued by either *EcRA*, *EcRB1* or *EcRB2* transgene expressed under the control of a heat shock promoter. The rescue of different alleles was dependent on the amount of EcRB protein synthetized in the mutant. The weakest hypomorph allele (*P-5738*) was rescued most effectively by a single heat shock at the end of L3 stage, whereas the strongest alleles (*P-25, P40*) needed a second heat shock in prepupal stage. In contrast *P-9* allele, which is similar hypomorph for *EcRB1* and *EcRB2* as *P-25* and *P-40*, but wild type for *EcRA* and *EcRC*, is needed only a single heat shock. This result underlies that the redundant EcRA protein participates in the processes during metamorphosis.

7. During the molecular characterisation of P-insertion *EcR* alleles we found examples for a general phenomenon which explains how transposone insertions in introns can inactivate the gene expression. The selection marker gene carried by the transposone can disturb mRNA processing if integrated in the same orientation as the affected gene. Abnormal excision of introns and fusion of exons of the affected gene with exons of the selection marker gene occurs and a chimeric mRNA is produced.

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The *Drosophila L(3)DTS-3 gene* encoding a Zn-finger protein regulates ecdysone biosythesis (poster) 2002

17th European Drosophila Research Conference, Edinburgh, Scotland
O. Komonyi, W. Girma, I. Kerekes, M. Mink, P. Maroy: The *DTS-3* gene encoding a C_2H_2 Zn-finger protein is essential in larval ecdysone production in *Drosophila* (poster) 2001

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O. Komonyi, W.Girma, I. Kerekes, M. Mink, P. Maroy: A new C_2H_2 Zn-finger protein with essential function in *Drosophila* ecdysone production
(oral presentation) 2001
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(oral presentation) 2001

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