발생과 생식 제 5권 제 2호, 131~136 (2001)
Dev. Reprod. Vol. 5, No. 2, 131~136 (2001)

Expression of *rpr*, *grim*, *dcp-1*, *diap1*, and *diap2* during *Drosophila* Development

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노랑초파리 발생과정에서 *rpr*, *grim*, *dcp-1*, *diap1*, *diap2*의 발현

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ABSTRACT : The developmental profiles of *rpr, grim, dcp-1, diap1, diap2* transcripts, which were involved in programmed cell death, were analyzed using competitive RT-PCR in whole animals during *Drosophila* development. The fluctuation patterns of transcript levels of the apoptotic initiators(*rpr* and *grim*) were similar to those of the ecdysone titer in *Drosophila* life cycle. The transcript of *dcp-1*, which is considered as effector caspase, was expressed strongly at early embryo and female adult stages. However, the transcript levels of anti-apoptotic factors *diap1* and *diap2*, showed the reverse pattern comparing with those of apoptotic factors(*rpr* and *grim*). Also, the transcript levels of *rpr*, *diap2* and *dcp-1* were quantified in the salivary glands and wing discs dissected from the wandering late third instar larva. The transcript levels of *rpr* and *diap2* were changed reversely each other in both tissues from wandering stage to puparium formation. These results suggest that the expressions of cell death related genes are regulated by the ecdysone signals during normal development.

Key words : Programmed cell death, *Drosophila*, Competitive RT-PCR, *rpr*, *grim*, *dcp-1*, *diap1*, *diap2*.

요 약 : 초파리 발생과정에서 세포사멸에 관여하는 유전자인 *reaper(rpr), grim, dcp-1, diap1, diap2*의 발현양상을 경쟁적 RT-PCR 방법으로 분석하였다. 세포사멸 유도 유전자인 *rpr, grim*의 발현양상은 발생단계에 따른 ecdysone titer 변화 양상과 매우 유사하였다. Effector caspase인 *dcp-1* 전사체는 초기 배와 암컷 성체에서 높은 발현을 보였다. 반면에 세포사멸 억제인자인 *diap1*과 *diap2* 전사체는 세포사멸 유도 인자인 *rpr*과 *grim* 전사체와 서로 상반적인 양상으로 발현되었다. 또한, 유주 3령 유충의 발생단계 별로 침샘조직과 성체원기조직에서 *rpr, diap2, dcp-1*의 전사체의 양적 변동을 분석하였다. *rpr, diap2*의 전사체양은 두 조직에서 서로 상반적으로 변화하였다. 이 결과는 정상 발생과정에서 세포죽음 관련유전자들의 발현이 ecdysone 신호에 의해 조절됨을 암시해 주었다.

INTRODUCTION

In *Drosophila*, the induction of apoptosis requires the products of *reaper(rpr)*, *hid*, and *grim* gene(White et al., 1994; Grether et al., 1995; Chen et al., 1996). RPR, HID, and GRIM share no extensive homology, although there is exception between the sequences of the N-terminal 14 amino acids of these proteins (Chen et al., 1996). These genes were expressed in regions of the embryo where cell death occurs and ectopic expression of these genes in the *Drosophila* retina resulted in eye ablation (Grether et al., 1995; Chen et al., 1996; White et al., 1996). Also, ectopic expression of *rpr* and *grim* induced the apoptosis in mammalian system, which transiently transfected into human breast carcinoma cells and mouse fibroblasts (Claveria et al., 1998; McCarthy & Dixit, 1998).

Apoptosis is negatively regulated by the inhibitor of apoptosis proteins(IAPs) (Deveraux & Reed., 1999). In *Drosophila*, two IAP molecules have been reported, which are *diap1* and *diap2* (Hay et al., 1995). These molecules were evolutionarily conserved with other IAP molecules, including the v-IAPs and mammalian IAPs(Hay et al., 1995; Deveraux & Reed., 1999). Overexpression of DIAPs in the eye suppressed normally occurring cell death as well as death induced by overexpression of *rpr* or *hid*. Also, DIAPs blocked the apoptosis induced by

This study was supported by the grant from Korea Research Foundation (1998).

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overexpression of HID and GRIM in the SF-21 cell line and physically interacted with them through the BIR motifs of the IAPs(Hay et al., 1995; Vicic et al., 1998).

Several caspases, which are considered to be essential effectors of apoptosis, have been identified and characterized in *Drosophila*(Song et al., 1997; Chen et al., 1998; McCall & Steller, 1998; Colussi et al., 2000; Hawkins et al., 1999; Quinn et al., 2000). Of these caspases, DCP-1 is considered to effector caspase, because this has the shorter prodomain and shows higher homology to mammalian effector caspases(Bangs & White, 2000). *dcp-1* $^{-}$ mutant show a defect in transfer of nurse cell cytoplasmic contents to developing oocytes, suggesting that *dcp-1* may be required for *Drosophila* oogenesis(McCall & Steller, 1998).

During the metamorphosis of *Drosophila*, a complex series of developmental programs is initiated that leads to a total reorganization of the body plan. These divergent morphogenetic pathways including apoptosis are initiated by increase in the titer of steroid hormone 20-hydroxyecdysone and juvenile hormone (Jiang et al., 1997; 2000).

In this study, to analyze the correlation between ecdysone titer fluctuation and expression of *rpr*, *grim*, *diap1*, *diap2*, *dcp-1* during *Drosophila* development, the transcript levels of these genes were analyzed using competitive RT-PCR in the whole animals and third instar larval tissues. We showed that the expressions of these genes were closely correlated with the fluctuation of ecdysteroids titer during normal development.

MATERIALS AND METHODS

1. *Drosophila* culture and developmental staging

A wild type Canton-S strain of *Drosophila melanogaster* was raised at 25℃ on standard medium(Lewis, 1960). Several hundred adult flies were transferred to 100mm Petri plates containing an apple medium at 25℃. Embryos were collected at 6h intervals after the eggs have been laid. For the larval stage, the early first instar larva were collected from agar plate, transferred to standard cornmeal media, and allowed to develop to appropriate stage at 25℃. Third instar larvae were staged by growth on food containing 0.1% bromophenol blue according to the method of Andres & Thummel(1994). For the pupal stage, the wandering third larva were transferred to a bottle containing cornmeal media, and allowed to develop at 25℃ until they reached the desired stages. Newly enclosed adult flies were collected and transferred to new bottles and incubated for 5 day. The salivary glands and wing discs from third instar larva were dissected in insect Ringers. The dissected tissues and staged animals were stored in liquid nitrogen until required for RNA extraction.

2. RNA isolation

Total RNA was extracted by using the RNA Zol B(Tel-Test) according to the manufacturer's protocol. Recovered RNA were re-extracted to eliminate contaminated DNA by treatment of RNase-free DNase(Promega). To calculate the amount of RNA, the absorbance was measured at 260nm and 280nm. The RNA samples with an A260/A280 ratio from 1.7～2.0 were used in the cDNA synthesis reaction.

3. Competitive RT-PCR

The gene-specific primer sets used in this study were shown in Table 1. Competitive RT-PCR was performed by the method of Kim et al.(1999). The first strand of cDNA was synthesized with 1 μg of total RNA, using the Advantage^{TM}-RT-for-PCR Kit(Clontech) according to manufacturer's protocol. After the cDNA synthesis reaction, it was diluted to final volume of 100 μl by adding 80 μl of DEPC-treated water. Five μl of the diluted cDNA were usually used for each 50 μl PCR reaction.

To construct an heterologous DNA competitor, appropriate sizes of *v-erbB* DNA fragment flanked by primer-binding sites (Table 1) were prepared so that amplified products from competitor and target cDNA could be discriminated by gel electrophoresis(PCR MIMIC Construction Kit, Clontech). Purified DNA competitors were quantified and diluted as competitive internal standards in PCR amplifications. PCR was performed in a DNA Thermal Cycler according to the following protocol: 5 μl of 10×PCR buffer, 3 μl of 25mM $MgCl_2$, 1 μl of dNTP mix (each 10mM), 0.5 μl of each primer sets(20 μM), 2 units of Taq DNA polymerase, and sterile deionized water to achieve a final volume of 50 μl. The amplification was performed for 35 cycles with 45 sec denaturation at 94℃, 45 sec annealing at 60℃, and 90 sec extension at 72℃. The resulting PCR products were analyzed on 1.6% agarose gels, and quantified using BioDoc Ⅱ gel video system(Biometra). The relative amount of cDNA in

Table 1. Cell death related gene primer sets used in RT-PCR

Gene	Primer sequence	Product size	Reference
rpr	5'-CTTGCGGGAGTCACAGTGGAGATTC-3' 5'-GCTCTGTGTCCTTGACTGCAGTAGA-3'	274bp	White et al., 1994
grim	5'-GCCCAATTGTTGGCCAGAAGCTATC-3' 5'-GTTCTCCTTGGAGGTGGCATCGGTA-3'	384bp	Chen et al., 1996
diap1	5'-CCGACTGGCCGCTAGATTGGCTGGA-3' 5'-GCGGTTCCAGTTACACTGCCTGCCG-3'	385bp	Hay et al., 1995
diap2	5'-ACGGAGCTGGGCATGGAGCTGGAGA-3' 5'-TTGCCAGAGCCTGCGGCGTGATGTT-3'	399bp	Hay et al., 1995
dcp-1	5'-ACCGACGAGTGCGTAACCAGAAACT-3' 5'-TGCTTCAGGATGTCCCTCAACTTGC-3'	392bp	Song et al., 1997
rp49	5'-GTGTATTCCGACCACGTTACA-3' 5'-TCCTACCAGCTTCAAGATCAC-3'	460bp	O'Conneall & Rosbath, 1984

the PCR mixtures was approximately estimated, on the assumption that the molar ratio of the PCR products reflects the initial molar ratio of the cDNA and the competitor.

RESULTS AND DISCUSSION

In preliminary experiments, we determined the optimal amount of RNA for cDNA synthesis and the optimal amount of cDNA template for following RT-PCR. Fig. 1 shows conventional RT -PCR results for the transcripts analysis of cell death related genes in whole animal extracts during *Drosophila* developmental stages. The predicted sizes of the RT-PCR products using gene-specific primers described in Table 1 were consistent with the sizes of the products in ethidium bromide stained agarose gels. Thus, the relative amounts of the transcripts of cell death related genes were quantified by competitive RT-PCR as shown in Fig. 2.

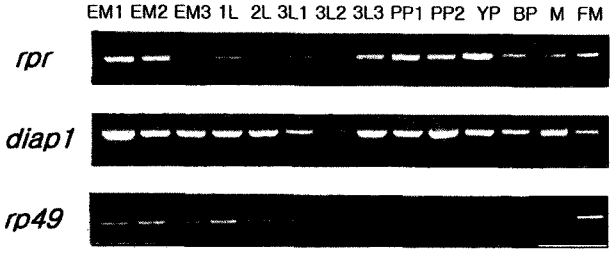


Fig. 1. RT-PCR assay of the transcripts of cell death related genes during Drosophila developmental stages. The *rp49* transcripts were controls for the RNA extraction and for subsequent RT-PCR. The columns represent the representative RT-PCR photography of the transcripts of *rpr*, *diap1*, and *rp49*, respectively. The lanes are labeled with the following abbreviations: EM1, early developed embryo; EM2, middle developed embryo; EM3, late developed embryo; 1L, first instar larvae; 2L, second instar larvae; 3L1, early developed third instar larvae; 3L2, middle developed third instar larvae; 3L3, late developed third instar larvae; PP1, early developed prepupae; PP2, late develped prepupae; YP, yellow pupae; BP, black pupae; M, adult male; FM, adult female.

1. Expression of *rpr*, *grim*, *diap1*, *diap2*, and *dcp-1* during development

The relative amounts of *rpr*, *grim*, *diap1*, *diap2*, and *dcp-1* transcripts measured in each developmental stage were shown in Fig. 3. The expression of *rpr*, *grim* were moderately high in the early embryos, highest in middle embryos, and decreased remarkedly to low level in late embryos. The transcriptional activities began to increase gradually from larval stages to prepual stages, reached peak in middle pupal stage, and then decreased through following stages(Fig. 3B). It was reported that there were three high-titer pulses of ecdysteroids during *Drosophila* development(Fig. 3A); firstly a midembryonic pulse, secondly, late-larval pulse that peaks at the larval-to-prepupal transition(pupariation), and thirdly, prepupal pulse that peak at 10~12 hr after puparium formation and causes head eversion and the prepupal-to-pupal transition(Richards, 1981; Sliter & Gilbert, 1992). Since the fluctuation patterns of *rpr* and *grim* transcript levels mimic those of the ecdysteroid titer, it was

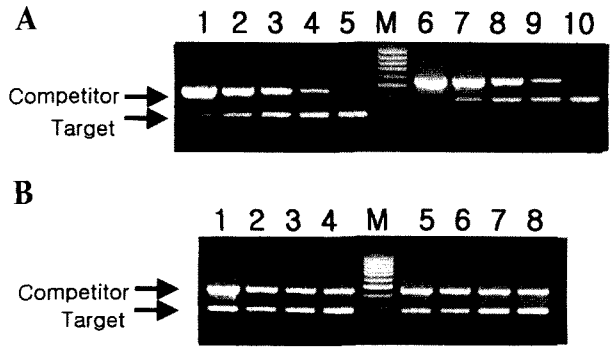
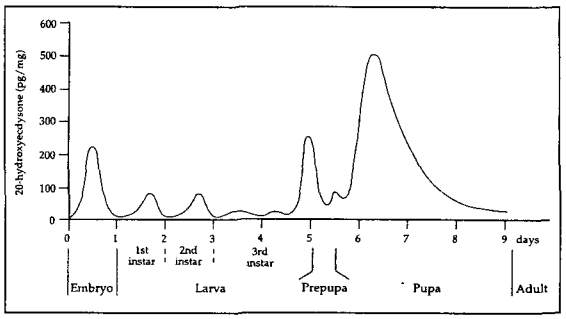


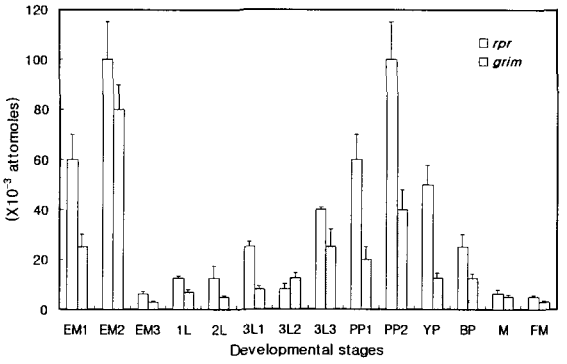
Fig. 2. Representative competitive RT-PCR assays to quantify the transcripts of cell death related genes. (A) The results of preliminary PCR of *rpr* and *grim* in late embryo stage. Lanes 1~5 and lanes 6~10 contain PCR products using *rpr* specific primer and *grim* specific primer, respectively. And, lanes 1~5 and lanes 6~10 contain a ten-fold serial dilution of the indicated MIMIC DNA($1\sim10^{-4}$ attomoles). (B) The fine-tuned PCR of *rpr* in midde and late embryo stage. Lanes 1~4 and lanes 5~8 contain PCR products using *rpr* specific primer. And, lanes 1~4 and lanes 5~8 contain a two-fold serial dilution of the indicated MIMIC DNA($2.5\times10^{-2}\sim3.125\times10^{-3}$ attomoles). One attomole is equal to approximately 600,000 molecules.

suggested that the expressions of these genes were mediated by ecdysteroid signal *in vivo*.

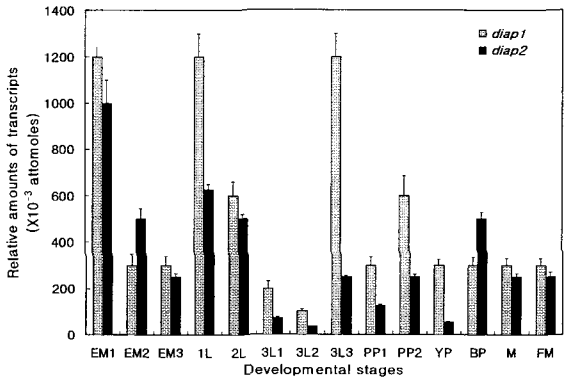
The expression of *diap1* and *diap2* transcripts were maintained at high level during first and second instar larval stages, in which *rpr* and *grim* transcripts were maintained at low level(Fig. 3C), suggesting that *diap1* and *diap2* play a role as anti-apoptotic factors in cell death caused by *rpr* and *grim*. The level of *dcp-1* transcripts was high in early embryo and adult females, comparing with those of other developmental stage(Fig. 3D). This result is consistent with *dcp-1* was expressed highly in preblastoderm embryos(Song et al., 1997) and oocytes(McCall & Steller, 1998).



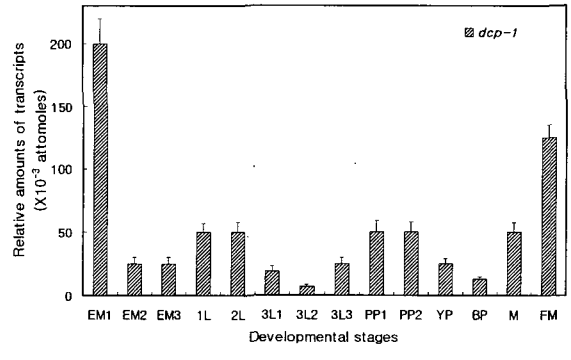
(A)



(B)



(C)



(D)

Fig. 3. The transcripts titers of cell death related genes in whole body extract during *Drosophila* life cycles. The relative amounts of *rpr* and *grim*(B), *diap1* and *diap2*(C), *dcp-1*(D) transcripts are shown below (A), the ecdysteroid titer profile during *Drosophila* development (Richards, 1981). The bars represent average values ± SD of samples from three separate experiments. The samples are labeled with the following abbreviations: EM1, early developed embryo; EM2, middle developed embryo; EM3, late developed embryo; 1L, first instar larvae; 2L, second instar larvae; 3L1, early developed third instar larvae; 3L2, middle developed third instar larvae; 3L3, late developed third instar larvae; PP1, early developed prepupae; PP2, late develped prepupae; YP, yellow pupae; BP, black pupae; M, adult male; FM, adult female.

2. Expression of *rpr*, *diap2*, and *dcp-1* in larval salivary glands and imaginal wing discs

The relative amounts of *rpr*, *diap2*, and *dcp-1* transcripts were analyzed in salivary glands and imaginal wing discs dissected from the wandering late third instar larva. In the salivary gland that potentionally will be undergo histolysis during metamorphosis, the level of *rpr* transcript was gradually increased, while the level of *diap2* transcript was gradually decreased(Fig. 4). However, in the imaginal wing disc that potentially will be differentiate to adult organ during metamorphosis, the level of *rpr* transcript was gradually decreased, while the level of *diap2* transcript was gradually increased(Fig. 4). The level of *dcp-1* transcript showed no significant difference in both tissues(Fig. 4). The opposite expression patterns of *rpr* and *diap2* in both tissues suggest that tissue-specific cell death around the onset of metamorphosis are controlled coordinately by initiators and inhibitors of cell death pathway, which are induced by several signals, including the change of ecdysteroid titer.

Though it is not exactly known how to produce a variety of tissue- and stage-specific response by a single hormone, this might be done through the use of different tissue- and stage -specific ecdysone receptors(Bender et al., 1997). It is interesting that *rpr* transcription is induced directly by the ecdysone -receptor complex through at least one EcRE in the *rpr* promotor, and *Broad-Complex*(BR-C) is required for both *rpr* and *hid* transcription(Jiang et al., 2000). Additionally, *diap2* induction is dependent on *β FTZ-F1*, while E75A and E75B are each sufficient to repress *diap2*(Jiang et al., 2000). These reports combined with our results suggest that ecdysteroid-inducible genes may regulate the expression of genes involved in cell death pathway.

In summary, our competitive RT-PCR analysis showed that the expressions of *rpr*, *grim*, *diap1*, *diap2*, and *dcp-1* were closely correlated with the fluctuation of ecdysone titer during normal development. Thus, it is required further study on the transcriptional regulators of the programmed cell death genes, which are expressed by ecdysteroid signal.

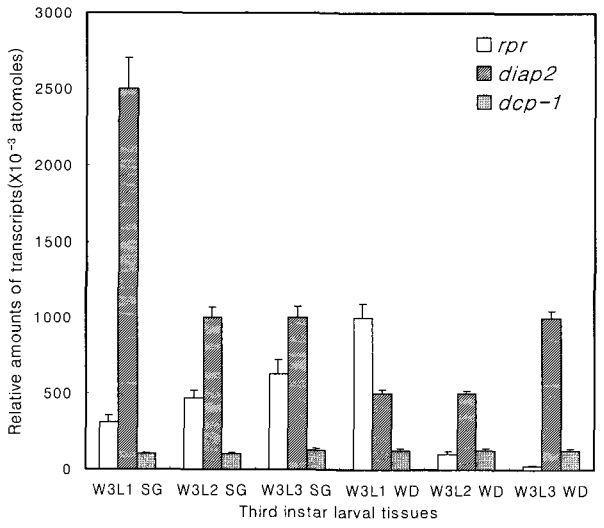


Fig. 4. The transcripts titers of *rpr*, *diap2*, and *dcp-1* in the salivary gland and imaginal wing disc from wandering third instar larvae to puparium formation. The bars represent average values ± SD of samples from three separate experiments. The samples are labeled with the following abbreviations: W3L1 SG, salivary gland from early wandering larvae; W3L2 SG, salivary gland from middle wandering larvae; W3L3 SG, salivary gland from larvae of puparium formation stages; W3L1 WD, wing discs from early wandering larvae; W3L2 WD, wing discs from middle wandering larvae; W3L3 WD, wing discs from larvae of puparium formation stages.

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