rigor mortis encodes a novel nuclear receptor interacting protein required for ecdysone signaling during *Drosophila* larval development

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Summary

Pulses of the steroid hormone ecdysone trigger the major developmental transitions in *Drosophila*, including molting and puparium formation. The ecdysone signal is transduced by the EcR/USP nuclear receptor heterodimer that binds to specific response elements in the genome and directly regulates target gene transcription. We describe a novel nuclear receptor interacting protein encoded by *rigor mortis* (*rig*) that is required for ecdysone responses during larval development. *rig* mutants display defects in molting, delayed larval development, larval lethality, duplicated mouth parts, and defects in puparium formation – phenotypes that resemble those seen in *EcR*, *usp*, *E75A* and *βFTZ-F1* mutants. Although the expression of these nuclear receptor genes is essentially normal in *rig* mutant larvae, the ecdysone-triggered switch in *E74* isoform expression is defective. *rig* encodes a protein with multiple WD-40 repeats and an LXXLL motif, sequences that act as specific protein-protein interaction domains. Consistent with the presence of these elements and the lethal phenotypes of *rig* mutants, Rig protein interacts with several *Drosophila* nuclear receptors in GST pull-down experiments, including EcR, USP, DHR3, SVP and βFTZ-F1. The ligand binding domain of βFTZ-F1 is sufficient for this interaction, which can occur in an AF-2-independent manner. Antibody stains reveal that Rig protein is present in the brain and imaginal discs of second and third instar larvae, where it is restricted to the cytoplasm. In larval salivary gland and midgut cells, however, Rig shuttles between the cytoplasm and nucleus in a spatially and temporally regulated manner, at times that correlate with the major lethal phase of *rig* mutants and major switches in ecdysone-regulated gene expression. Taken together, these data indicate that *rig* exerts essential functions during larval development through gene-specific effects on ecdysone-regulated transcription, most likely as a cofactor for one or more nuclear receptors. Furthermore, the dynamic intracellular redistribution of Rig protein suggests that it may act to refine spatial and temporal responses to ecdysone during development.

Key words: Nuclear receptor, Hormone, Cofactor, Ecdysone, Molting

Introduction

The nuclear receptor superfamily of ligand-dependent transcription factors plays a central role in coordinating the development and physiology of higher organisms. Nuclear receptors are defined by two functional domains, a highly conserved DNA binding domain and a less conserved ligand binding domain that provides a ligand-dependent activation function. Nuclear receptors exert their transcriptional effects through direct interactions with specific cofactors, including the p300/CBP and SRC family coactivators and NCoR and SMRT corepressors (Glass and Rosenfeld, 2000; Dilworth and Chambon, 2001). These cofactors contain multiple functional domains and, in some cases, have enzymatic activities that allow them to directly modify the chromatin template, resulting in the appropriate induction or repression of target gene expression.

The fruit fly, *Drosophila melanogaster*, provides an ideal model system for defining the molecular mechanisms of hormone action in the context of an intact developing organism. Progression through the *Drosophila* life cycle is dependent on pulses of the steroid hormone ecdysone that direct the major developmental transitions, including molting and puparium formation (Riddiford, 1993). Ecdysone exerts its effects through a heterodimer of two members of the nuclear receptor superfamily, the EcR ecdysone receptor (NR1H1) and the fly RXR ortholog USP (Ultraspiracle, NR2B4) (Riddiford et al., 2001). These receptors are widely expressed, allowing the transduction of the systemic ecdysone signal throughout development (Talbot et al., 1993; Henrich et al., 1994). Upon binding ecdysone, the EcR/USP heterodimer directly induces target gene expression, including a small set of early regulatory

genes that were originally identified as ecdysone-inducible puffs in the larval salivary gland polytene chromosomes (Ashburner et al., 1974; Thummel, 1996; Richards, 1997; Henrich et al., 1999; Riddiford et al., 2001). These include *E74*, which encodes two related proteins that share a C-terminal ETS DNA binding domain, E74A and E74B (Burtis et al., 1990), and *E75*, which encodes three orphan members of the nuclear receptor superfamily, E75A, E75B and E75C (NR1D3) (Segraves and Hogness, 1990). The early ecdysone-inducible transcription factors coordinate the expression of numerous secondary-response late genes that are responsible for directing appropriate stage- and tissue-specific biological responses during development (Thummel, 1996; Richards, 1997; Henrich et al., 1999; Riddiford et al., 2001).

Mutations in EcR and USP lead to a range of phenotypes that reflect the requirements for ecdysone signaling during development. *EcR* mutants display defects and delays in larval molting, with some animals arresting development at the molts (Bender et al., 1997; Schubiger et al., 1998; Li and Bender, 2000). Many of these mutants retain the cuticle and mouthhooks from the preceding instar. Animals that survive to the third instar are significantly delayed and form elongated prepupae that retain their larval morphology. Most of these phenotypes are shared by *usp* mutants, consistent with the idea that EcR and USP act together as a heterodimeric ecdysone receptor (Perrimon et al., 1985; Oro et al., 1992; Hall and Thummel, 1998). Molting defects are also seen in animals that carry mutations in the *E75A* and *βFTZ-F1* (NR5A3) ecdysone-regulated orphan nuclear receptor genes (Yamada et al., 2000; Bialecki et al., 2002). *E75A* and *βFTZ-F1* mutants die with duplicated cuticles, mouth parts and anterior spiracles, indicating an inability to complete the molting cycle. For *E75A* mutants, these defects are caused by a reduced ecdysone titer, implicating a key role for this receptor in hormone biosynthesis or release (Bialecki et al., 2002).

We describe a novel nuclear receptor interacting protein encoded by *rigor mortis* (*rig*) that is required for the ecdysone-regulated processes of molting and puparium formation. Rig acts downstream from ecdysone biosynthesis and release to control the expression of specific ecdysone-regulated genes and it interacts directly with several *Drosophila* nuclear receptors, including EcR, USP and βFTZ-F1. Antibody stains reveal that Rig is localized to the cytoplasm of imaginal cells and neuroblasts, but shuttles in and out of the nuclei in the larval salivary glands and midguts in a spatially and temporally restricted manner. Taken together, these data suggest that Rig is an integral part of the ecdysone signaling cascade, acting as a novel cofactor for one or more *Drosophila* nuclear receptors.

Materials and methods

rig mutant characterization

Fly stocks *l(2)05056*, *l(2)k07839* and *l(2)k07917* (Torok et al., 1993) were supplied by the Berkeley *Drosophila* Genome Project (BDGP). *l(2)05056* and *l(2)k07917* are available from the Bloomington Stock Center. *l(2)k07839*, *l(2)k07917*, and the deficiency *Df(2R)exu*^{1}, were maintained over a *CyO y*$^{+}$ chromosome in a *y w* background. Animals transheterozygous for various combinations of the P-element or deficiency chromosomes were identified by the yellow phenotype of their mouthhooks and denticle belts. Unless otherwise specified, *y w* animals were used as the control. The degree of embryonic lethality associated with each transheterozygous combination of the P-element and deficiency chromosomes was determined by collecting embryos from the cross *y w*; *l(2)k07839* or *l(2)k07917* or *Df(2R)exu*^{1}/*CyO y*$^{+}$ × *y w*; *l(2)k07839* or *l(2)k07917* or *Df(2R)exu*^{1}/*CyO y*$^{+}$. For the control, embryos were collected from the cross *y w*; +/*CyO y*$^{+}$ × *y w*; +/*CyO y*$^{+}$. The total number of embryos was counted and maintained at 25°C. The number of dead embryos was determined after 2 days. To identify lethality at later stages of development, embryos were collected from the above cross and maintained at 25°C. Mutant first instar larvae were selected on the basis of the yellow phenotype of their mouthhooks and denticle belts and placed on fresh yeast paste in a petri dish lined with damp black Whatman paper. The larvae were maintained at a density of approximately 50 animals per petri dish at 25°C. The animals were removed from the yeast paste at least every 24 hours, phenotyped and placed on fresh yeast paste in a new petri dish until all animals had died. A similar range of lethal phenotypes was observed with lines derived from *l(2)k07917* and *l(2)k07839* that had been subjected to free recombination for three generations, arguing that the lethality in these lines is linked to the P-element insertion (data not shown). DNA adjacent to the *l(2)k07839* P-element insertion was recovered by plasmid rescue as described previously (Hamilton and Zinn, 1994). The DNA sequence of LD12835 has been submitted to GenBank (accession number AY274835). Tests for dominant genetic interactions were conducted by assessing the viability of animals carrying one mutant allele of *rig* (*l(2)k07917*, *l(2)k07839* or *Df(2R)exu*^{1}) and one mutant allele of each receptor: *EcR*$^{l(2)k06210}$ (D'Avino and Thummel, 2000), *EcR*^{C300Y}, *EcR*^{M554fs} (Bender et al., 1997), *usp*^{3}, *usp*^{4} (Oro et al., 1992), *βFTZ-F1*^{ex17}, *βFTZ-F1*^{ex19} (Broadus et al., 1999), *E75*^{Δ51} and *E75*^{A81} (Bialecki et al., 2002). We also tested for recessive genetic interactions using either *l(2)k07917 or Df(2R)exu*^{1} and *βFTZ-F1*^{ex17}/ *βFTZ-F1*^{ex17}, or examining *l(2)k07917*, *EcR*^{M554fs}/*Df(2R)exu*^{1} or *l(2)k07917*, *EcR*^{M554fs}/*EcR*^{Q50st} animals.

Ecdysone feeding experiments

Embryos from the cross *y w*; *l(2)k07839*/*CyO y*$^{+}$ × *y w*; *Df(2R)exu*^{1}/*CyO y*$^{+}$ were collected for 3 to 4 hours. As controls, embryos from the crosses *y w*; *Df(2R)exu*^{1}/*CyO y*$^{+}$ × *y w* or *E75*^{A81}/TM3, GFP × *E75*^{Δ51}/TM3, GFP were collected for 3 to 4 hours. The embryos were maintained at 25°C and allowed to hatch. *l(2)k07839*/*Df(2R)exu*^{1} and +/*Df(2R)exu*^{1} first instar larvae were selected by the yellow phenotype of their mouthhooks and denticle belts. *E75*^{A81}/*E75*^{Δ51} first instar larvae were selected by their lack of GFP expression. First instar larvae were placed on fresh yeast paste in a petri dish lined with damp black Whatman paper at 25°C. Larvae were allowed to develop until 66 hours after the beginning of the egg lay, a time point corresponding to 6 hours before the molt to the third instar. To make yeast paste containing 0.5 mg/ml 20-hydroxyecdysone (Sigma), 4.2 μl of a 12 mg/ml stock solution (in ethanol) was diluted in 95.8 μl water and added to 0.05 g of dry yeast. As a negative control, 4.2 μl ethanol was diluted in 95.8 μl water and added to 0.05 g of dry yeast. The staged second instar larvae were transferred to these yeast samples, allowed to feed for 6 hours, and then returned to regular yeast paste. The animals were scored 18-24 hours later, a time corresponding to the middle of the third instar in wild-type animals.

Northern blot hybridizations

RNA was isolated from staged +/*Df(2R)exu*^{1} control or *l(2)k07839*/*Df(2R)exu*^{1} mutant second and third instar larvae by direct phenol extraction as described previously (Andres and Thummel, 1994). Animals were staged at 6-hour intervals from the first-to-second instar molt, and again at the second-to-third instar molt for two time points. Equal amounts of RNA from each stage were fractionated by formaldehyde agarose gel electrophoresis and transferred to a nylon membrane (Genescreen; Dupont) as described previously (Karim and Thummel, 1991). A probe to detect *rig* transcription was generated by PCR amplification of bases 910-1400 from the cDNA LD12835. The remaining probes were generated as described

previously (Karim and Thummel, 1991; Andres et al., 1993). All DNA fragments were gel purified (Geneclean; Bio101) and labeled by random priming (Prime-it Kit; Stratagene). The blots were hybridized, washed and stripped as described previously (Karim and Thummel, 1991).

In vitro protein binding assays

cDNAs for EcR, FTZ-F1, DHR3, USP and SVP were fused to the coding region for GST in the pGEX-2T plasmid vector (Pharmacia) as described previously (Beckstead et al., 2001) and expressed at high levels in bacteria. For expression of ^{35}S-labelled Rig, the complete coding sequence was inserted into the pSG5 vector (Stratagene) and coupled transcription/translation was performed using T7 RNA polymerase with the TNT lysate system (Promega). GST and GST fusion proteins were expressed in *E. coli* and purified on gluthathione-Sepharose beads (Pharmacia), as described by the manufacturer. Purified proteins were quantified by Coomassie Blue staining after SDS-PAGE separation and Bradford protein assay. All proteins were of an appropriate length for the corresponding constructs. Glutathione-Sepharose beads were equilibrated with binding buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.3 mM DTT, 1 mM PMSF, 10 mM $MgCl_2$, 5% glycerol, 0.5% NP40, and protease inhibitor mixture), loaded with equimolar amounts of GST or GST fusions and washed as described by Beckstead et al. (Beckstead et al., 2001). For binding assays, 5 μl of ^{35}S-labelled Rig protein was incubated with 20 μl of bead-bound GST-fusion proteins for 1 hour at 20°C with gentle agitation in a final volume of 200 μl binding buffer. After three washes with 200 μl binding buffer, the beads were resuspended in 15 μl SDS-loading buffer, boiled for 5 minutes, and the bound proteins were analyzed by SDS-PAGE. Gels were dried, and radiolabeled Rig was detected by autoradiography.

Antibody stains

A region of *rig* encoding the C-terminal amino acids 862-1235 was amplified by PCR using the following primers: 5′-CAGAATTCCGACATTAAGGACGCGCTGGA-3′ and 5′-ACGGTCGACTTAATGCTCGGCAGTAGATC-3′. This fragment was purified, cut with *Eco*RI and *Sal*I and inserted into pGEX-5X-3 (Amersham). Purified GST-Rig was injected into three rabbits (Cocalico Biologicals Inc.) and antisera was screened at a 1:500 dilution by western blotting (ECL, Amersham Biosciences) using transformants that overexpress Rig under the control of the *hsp70* promoter (*hs-rig*). Antiserum was centrifuged and the supernatant was passed over two columns to affinity-purify anti-Rig antibodies, as described by Carroll and Laughon (Carroll and Laughon, 1987). The first column contained a protein extract from pGEX-5X-3 bacteria and the second column contained purified GST-Rig. Specific anti-Rig antibodies were eluted from the second column and dialyzed into PBS. Each fraction (~0.5 ml) was tested at a 1:500 dilution by western blot analysis using protein isolated from pGST-Rig bacteria and Rig overexpressed from *hs-rig* transformants. For detecting Rig protein in larval and prepupal organs, second and third instar w^{1118} larvae were staged from the molt. Animals were dissected and stained with affinity-purified anti-Rig antibody at a dilution of 1:100, using 1:200 Cy3-labeled anti-rabbit secondary antibodies (Jackson). Nuclei were detected by staining with a 1:1000 dilution of mouse anti-histone antibody (mAb052, Chemicon) and 1:1000 Cy2-labeled anti-mouse secondary antibodies (Jackson). The stains were imaged on a BioRad MRC1024 confocal laser scanning microscope using dual detector channels to independently visualize the Cy2 and Cy3 signals.

Results

rig mutants die at ecdysone-triggered developmental transitions

The *l(2)05056* P-element insertion line was obtained in the course of studying *bancal*, an ecdysone-inducible gene identified in a genetic screen for defects in leg disc morphogenesis (Gates and Thummel, 2000). Although *l(2)05056* was originally reported as an allele of *bancal*, we found that this mutation complemented the *bancal* P-element allele, bl^{k08305}. In addition, two other P-element insertion lines, *l(2)k07917* and *l(2)k07839*, failed to complement *l(2)05056* and complemented bl^{k08305}. These data thus indicate that *l(2)05056*, *l(2)k07917* and *l(2)k07839* constitute a lethal complementation group that is distinct from *bancal* – a complementation group that we refer to here as *rigor mortis* or *rig*. Subsequent studies of *l(2)05056* revealed that it carries a second-site female sterile mutation on the second chromosome, leading us to exclude this line from further analysis and focus on *l(2)k07917* and *l(2)k07839*.

A lethal phase analysis was performed as a first step toward defining the functions of *rig* during development. Crosses were set up between *y w*; *l(2)k07917*/*CyO* y^+ or *y w*; *l(2)k07839*/*CyO* y^+ and a deficiency for the region, *y w*; *Df(2R)exu*^{1}/*CyO* y^+, to test for embryonic lethality among the offspring. Progeny from the stock *y w*; +/*CyO* y^+ were used as a control. These experiments revealed that 19-28% of the embryos from *rig* mutant crosses died (Table 1), a number attributable to lethality from the homozygous *CyO* balancer chromosome (Table 1, control), indicating that zygotic *rig* function is not required during embryonic development.

rig mutant first instar larvae were collected at hatching and examined at regular intervals for phenotypes and lethality at later stages of development. The majority of *rig* mutants die as third instar larvae (Table 1). During the first day of the third instar, approximately half of the mutant animals stop moving and become extended and stiff in an apparent attempt to pupariate (Fig. 1A). These animals appear to survive in this immobile state for 2-3 days, after which patches of necrotic cells can be seen throughout the animal and the internal tissues begin to lose their integrity. Most of the remaining mutant third instar larvae become stationary over the next few days, while some stop eating and shrink in size until they finally die. Some of the mutants pupariate normally, although 1-5 days later than control animals, and then die as prepupae. The second most prominent lethal phase is during the second instar (Table 1). Of those *rig* mutants that die during this stage, approximately half display no apparent external defects. The other half survive two or more days past the time when they should have molted to the third instar and obtain the size of a late third instar, but retain the anterior spiracles of a second instar larva (compare Fig. 1B with 1E,F). These prolonged second instar larvae usually become stationary and die around the same time as their third instar siblings. The remaining *rig* mutants die while trying to molt to the third instar. These larvae have two sets of mouth parts and, in some cases, two sets of anterior spiracles and/or two cuticles (Fig. 1C,D, Fig. 2). *l(2)k07917*/*l(2)k07839* transheterozygotes display approximately the same range and frequency of lethal phenotypes as that seen when each mutant chromosome is maintained over a deficiency suggesting that these are strong loss-of-function alleles for the *rig* locus (Table 1).

Close inspection of *rig* mutant larvae revealed a defect in the formation of the third instar mouthhooks. Wild-type second instar larval mouthhooks have three to four large teeth (Fig. 2A), whereas third instar mouthhooks are larger

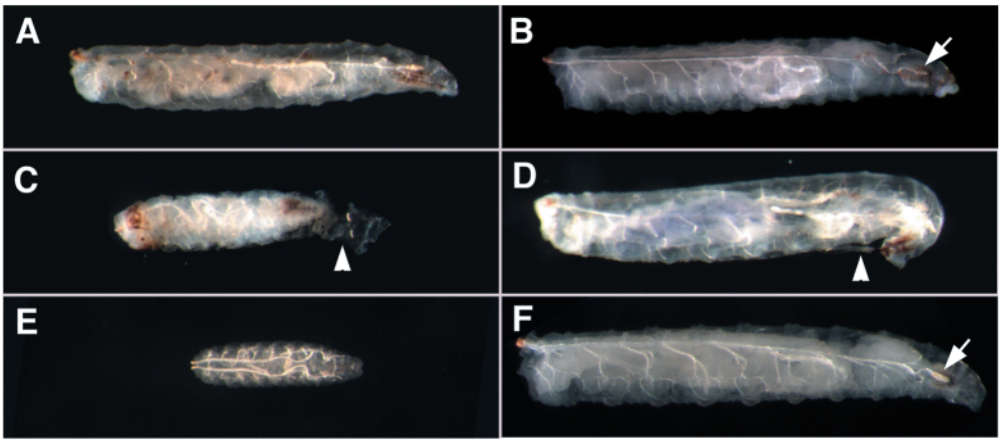

Fig. 1. Lethal phenotypes of *rig* mutant larvae. (A) The majority of *rig* mutant larvae (*l(2)k07839*/*Df(2R)exu*^{1}) die during the third instar and become stiff and extended in an apparent attempt to pupariate. (B) *rig* mutants that die as prolonged second instar larvae reach the size of a normal late third instar larva. The anterior spiracles of these prolonged second instar larvae end with a single club-like structure (arrow) rather than the multiple branches associated with a wild type third instar anterior spiracle (F, arrow). (C) Two cuticles are often seen when *rig* mutants die while molting. This larva died trapped inside the second instar cuticle, which had separated almost completely from the third instar cuticle (arrowhead). (D) This larva managed to shed its second instar cuticle from the anterior portion of its body, but died while trying to shed this cuticle further. A thin piece of cuticle extends from its mouthhooks to the point on the body where the second instar cuticle remains (arrowhead). (E,F) A *y w* early second instar larva (E) and *y w* late third instar larva (F) are shown as controls. All panels are lateral views with anterior to the right, except for E which is a dorsal view. All images were taken at the same magnification.

and have numerous smaller teeth (Fig. 2B). *rig* mutants that die as prolonged second instar larvae display relatively normal second instar mouthhooks (Fig. 2C). Mutant larvae that die during the third instar, however, have severely malformed mouthhooks that do not resemble those of either a second or third instar larva (Fig. 2D). *rig* mutants that die with molting defects have two sets of mouthhooks, a normal second instar set and a malformed third instar set (Fig. 2E).

Ecdysone feeding fails to rescue *rig* mutant animals

The defects in molting and puparium formation seen in *rig* mutants could result from either a decrease in the ecdysone titer or a decrease in the ability of the ecdysone signal to be transduced. To distinguish between these possibilities, we examined the effects of feeding ecdysone to *rig* mutant larvae. This method has been shown to effectively rescue phenotypes associated with ecdysone-deficient mutations (Garen et al., 1977; Venkatesh and Hasan, 1997; Freeman et al., 1999; Bialecki et al., 2002). Mid-second instar larvae were transferred to food either with or without 0.5 mg/ml 20-hydroxyecdysone (20E, the active form of the hormone) for 6 hours and scored 18-24 hours later, a time corresponding to the middle of the third instar in wild-type animals. Feeding ecdysone to control larvae (+/*Df(2R)exu*^{1}) reduces their viability as the number of active third instar larvae is decreased and the number of animals dying as second instar larvae or while molting to the third instar is increased (Table 2). This detrimental effect is most likely due to the excessive levels of hormone present in these animals. In contrast, little effect is seen when *rig* mutant larvae are exposed to the same hormone feeding regime. The number of animals dying during the second-to-third instar molt increased in *rig* mutants, but the overall range of lethal phenotypes was not significantly affected (Table 2). Feeding ecdysone to *E75A* mutant second instar larvae, however, had a dramatic effect

Table 1. Most *rig* mutants die during larval development

Genotypes	Embryo	*n*	1st instar	2nd instar	2nd prepupa	2nd/3rd instar	3rd instar	Prepupa	Pupa	*n*
$\frac{l(2)k07917}{Df(2R)exu^{1}}$	25%	100	0	2%	0	11%	69%	14%	4%	197
$\frac{l(2)k07839}{Df(2R)exu^{1}}$	28%	100	0	6%	1%	16%	68%	6%	3%	149
$\frac{l(2)k07917}{l(2)k07839}$	19%	100	1%	11%	0	11%	66%	11%	0	99
Control	25%	440	0	0	0	0	1	0	4%	97

The percentages of *rig* mutant animals that die at each stage in development are listed. To determine the degree of embryonic lethality, embryos were collected from the crosses *y w*; *l(2)k07839* or *l(2)k07917* or *Df(2R)exu/CyO y*$^{+}$ × *y w*; *l(2)k07839* or *l(2)k07917* or *Df(2R)exu/CyO y*$^{+}$ and the percentage of dead embryos was determined. For the control, embryos were collected from the cross *y w*; +/*CyO y*$^{+}$ × *y w*; +/*CyO y*$^{+}$. *n* refers to the total number of eggs collected. To determine the lethality caused by mutations in *rig* during later stages of development, mutant first instar larvae from the above crosses were selected and their development monitored. *n* indicates the total number of mutant larvae examined. The '2nd prepupa' class refers to animals that pupariate from the second instar. Animals that die while trying to molt from the second to the third instar are represented in the '2nd/3rd instar' class. *y w* animals were used as controls.

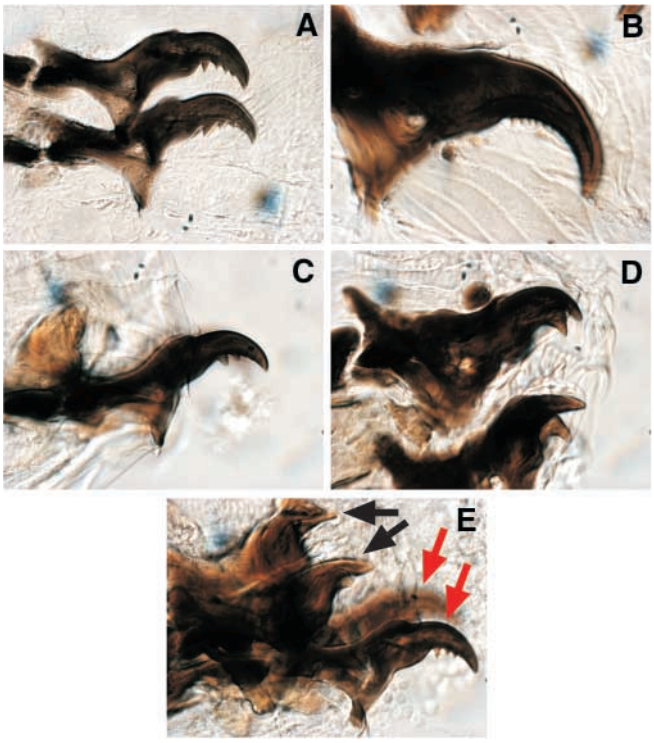

Fig. 2. *rig* mutant larvae have defective mouthhooks. (A) The mouthhooks of control (+/*Df(2R)exu*^{1}) second instar larvae have three to four large teeth, while control third instar larvae (B) have larger mouthhooks with numerous smaller teeth. (C) *rig* mutants (*l(2)k07839*/*Df(2R)exu*^{1}) that die as prolonged second instar larvae have normal second instar mouthhooks. (D) The mouthhooks of *rig* mutant that die as third instar larvae are severely malformed. (E) *rig* mutants that die while trying to molt have two sets of mouthhooks, a normal second instar set (red arrows) and a malformed third instar set (black arrows).

on their development, rescuing most of them to the third instar stage, consistent with the results of earlier work, and the identification of *E75A* mutants as ecdysone deficient (Table 2) (Bialecki et al., 2002). We conclude that ecdysone is not limiting in *rig* mutants and that *rig* functions downstream of ecdysone biosynthesis and release.

rig is required for ecdysone-regulated *E74* transcription

The earliest apparent defects in *rig* mutants occur during the second larval instar and molt to the third instar (Table 1), prompting us to examine ecdysone-regulated gene expression during these stages of development. RNA was isolated from second instar larvae staged at 6-hour intervals following the first-to-second instar molt (0, 6, 12, 18 hours, Fig. 3), immediately after the second-to-third instar molt (24 hours), and 6 hours later (30 hours) in both control (+/*Df(2R)exu*^{1}) and *rig* mutant (*l(2)k07839*/*Df(2R)exu*^{1}) animals. Equal amounts of total RNA were analyzed by northern blot hybridization to detect *rig*, *E75A*, *βFTZ-F1*, *E74*, *EcR*, and *usp* transcription (Fig. 3). A single mRNA, approximately 4.5 kb in length, was detected in control animals by hybridization with a radioactive *rig* probe. This transcript is present at a low constant level throughout the time course, with reduced levels at 0 and 18 hours, and is not detectable in *rig* mutant larvae, consistent with the identification of *l(2)k07839* as a strong loss-of-function *rig* mutation (Fig. 3).

We examined *EcR*, *usp*, *E75A* and *βFTZ-F1* expression in *rig* mutant larvae because mutations in these nuclear receptor genes result in phenotypes that resemble those seen in *rig* mutants (Oro et al., 1992; Bender et al., 1997; Hall and Thummel, 1998; Schubiger et al., 1998; Li and Bender, 2000; Yamada et al., 2000; Bialecki et al., 2002). Expression of these four genes, however, is essentially unaffected by the *rig* mutation (Fig. 3). There is a slight effect on *EcR* transcription, with reduced levels at 18 hours and a defect in the down-regulation that normally occurs following the molt to the third instar (Talbot et al., 1993; Sullivan and Thummel, 2003). In contrast, a significant effect is seen in *E74* expression in *rig* mutant larvae. Although no role for *E74* has been identified during larval stages (Fletcher et al., 1995), this gene acts as a sensitive indicator of ecdysone signaling, with low levels of hormone inducing *E74B* and higher levels of hormone repressing *E74B* and inducing *E74A* (Karim and Thummel, 1991). We see the normal very low expression of *E74B* in mid-second instar control larvae, a burst of *E74A* mRNA at 18 hours, and then reinduction of *E74B* in early third instar larvae (Fig. 3) (Sullivan and Thummel, 2003). In contrast, *E74B* is reinduced prematurely in *rig* mutants and then down-regulated while *E74A* transcription is reduced and delayed (Fig. 3), a

Table 2. Ecdysone feeding fails to rescue *rig* mutant phenotypes

		2nd instar		2nd/3rd instar	3rd instar			
		Active	Dead		Active	Extended	Dead	*n*
+ / *Df(2R)exu*^{1}	Control	0	0	0	100%	0	0	46
	20E	0	17%	22%	59%	0	2%	46
l(2)k07839 / *Df(2R)exu*^{1}	Control	2%	0	9%	61%	28%	0	46
	20E	0	0	24%	37%	39%	0	41
E75^{A81} / *E75*^{Δ51}	Control	88%	0	4%	4%	0	4%	28
	20E	0	14%	22%	57%	0	7%	28

Second instar larvae, staged 14-18 hours after the second-to-third instar larval molt, were fed yeast paste either with (20E) or without (control) 0.5 mg/ml 20-hydroxyecdysone for 6 hours and phenotyped 18-24 hours later. The percentages of animals observed at each stage 18-24 hours after feeding are shown. *n* refers to the total number of staged second instar larvae fed.

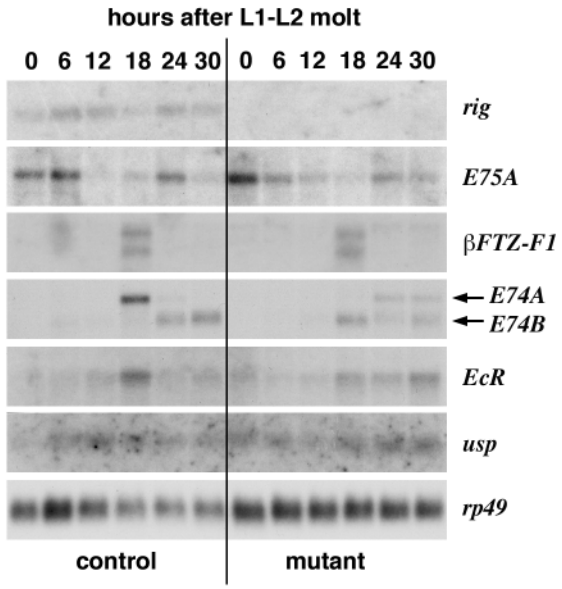

Fig. 3. *rig* exerts gene-specific effects on ecdysone-regulated gene expression. RNA was extracted from staged control (+/*Df(2R)exu*^{1}) and mutant (*l(2)k07839*/*Df(2R)exu*^{1}) second instar (0, 6, 12 or 18 hours after the first-to-second instar molt) larvae. Animals were re-synchronized at the molt to the third instar and RNA was collected either at the molt (24 hours after the first-to-second instar molt) or 6 hours later (30 hours). Equal amounts of total RNA were fractionated by formaldehyde agarose gel electrophoresis and analyzed by northern blot hybridization. Radiolabeled probes were used to detect *rig*, *E75A*, *βFTZ-F1*, *E74A*, *E74B*, *EcR*, and *usp* transcription. Hybridization to detect *rp49* mRNA was used as a control for loading and transfer.

pattern that is characteristic of reduced ecdysone signaling (Karim and Thummel, 1991). The observation that only some ecdysone-regulated genes are affected in *rig* mutants is consistent with the ecdysone feeding experiment and argues that *rig* exerts effects in a gene-specific manner. Identical patterns of transcription were seen using RNA samples isolated from a separate collection of control and mutant animals (data not shown).

rig encodes a WD-40 repeat protein with an LXXLL motif

Plasmid rescue was used to obtain 1,161 bp of DNA sequence flanking the *l(2)k07839* P-element insertion. This sequence overlapped with 692 bp of sequence flanking the *l(2)k07917* P-element insertion, reported by the Berkeley *Drosophila* Genome Project (BDGP) (Spradling et al., 1999), as well as a family of 21 overlapping cDNAs in the BDGP expressed sequence tag database (CG30149 on FlyBase, 2003). A representative of this family, LD12835, was sequenced. This cDNA is 4,120 bp long and contains a predicted open reading frame encoding a 1,235 amino acid protein. The coding region is preceded by an in-frame stop codon and followed by a poly(A) sequence, suggesting that it is full length. The length of the cDNA is also similar to the 4.5 kb *rig* mRNA detected by northern blot hybridization (Fig. 3). The P-element in both *l(2)k07917* and *l(2)k07839* is inserted into the 5′ untranslated region of the gene, 70 bp upstream from the predicted start codon. Analysis of the predicted Rig protein sequence reveals the presence of 7-12 WD-40 repeats, depending on the protein motif search engine used. For example, the Simple Modular Architecture Research Tool (SMART) predicts seven WD-40 repeats (Schultz et al., 2000), the Rep-V1.1 search tool identifies eight WD-40 repeats (Andrade et al., 2000) and the Protein Sequence Analysis tool recognizes 12 WD-40 repeats (http://BMERC-www.bu.edu/wdrepeat). The positions of nine of these WD-40 repeats are predicted by two or more of the above search engines and are highlighted in Fig. 4. BLAST searches using the Rig protein sequence did not reveal significant similarity to previously identified proteins outside of the WD-40 repeats. The Rig protein sequence also contains an LXXLL motif, a signature sequence found in many nuclear receptor cofactors (box, Fig. 4) (Heery et al., 1997; Torchia et al., 1997; Voegel et al., 1998; Glass and Rosenfeld, 2000).

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   1 MNSQVMYHNV PTPPGNVSLA AAAPDGGLLY AGIRCINYIS APPANGEQPQ VVTMSTRINI
  61 LALDVSPMWG LGNGGPTKPF AIVGDDLSVQ VWDCALGEAV IGHKAHQHQH EARDVRVVHH
 121 TTNSVLMSYL ANGNILSMDA SDLVIYCVAS NTYCRRSTFI SPRNHQLTMV RCSPYNDNLF
 181 AVGTAMGNVL VCDLRKMNIV YKFHGHKAPI CGLAWREVPA AEDEKTNNLA LSAEEWRSRN
 241 GGQEEKPKTK PPPLTKSKAA ESDDPFDIYN FDHLEYEFGA PIAERRRKSS EDCGGEFVGL
 301 EKPAGAAVLD FVEACESVKA ELLASRQEDK TQHVEVTLHD CEPTKPTGPL SDASTISNKN
 361 DASDSTEGSL EVIQYSSSSD DAVIVDGEAA KPKREVLHHI YHQAEVHASG TPQTKSEPQS
 421 NLQVVPAISA ETISLTSVNS THLETLLVSI DGDEVMMIWN TNTGAHAGKN YSKSKTAGKL
 481 NNVYWLNNHV IVSLSRHQLF FWSVEFERKM LRYKISKDKS HSCHLQDIVS FACDSSKEMI
 541 WLCRNNRQIG MMNPKTGRMA DFYGTVAFGV RAMAECPDDM NKIALGCSDR RVAFFDISKL
 601 TTSCLPIDSV YVSSNVYCLA WSPNCLELAF GTFDGTVGIL DVERMKVKTH LRTPHKKEVY
 661 SLVWQDHFIY FIVNRVLGFF DLRKSKIEPT IVNCISRPSY LSIRDSFLFV GTDDGLLQIH
 721 ERDSGMEKSW SPFIRQSALF ARYVTDIAWC PLDSNKFAVS GNDRSVYVME FQPTERNWKT
 781 LHTFTANTEK ASITSMRWSH TQKHLLLTFH IEGKVCLWNC NAPEKPPLTI TYHCPMWCGM
 841 FLPTNENIIM CSGKALSVEL IDIKDALEGD EKSICPKVDA LLNVKWASKS LTQPYAPVLT
 901 AAEKKRQRRD QRKAAAKLEV DVANKDQKKI QESVTAVIDN PTNDKCTQET PVEEMLEALS
 961 LDKEQNNRSA KECPKCKEQS PDSFTHSRTC LYLTQKELNK SALEKLAIVL TEDSAKIDKS
1021 VLISKLFSSK VMAKELIATE LTNLKHSNTK DIAPLCLAMS TFKLREELEQ HIANKTLTER
1081 HVSLAPSVSF TLWQDCCRAY AKQMEEKGYI MHAATYLFSQ GMQSEAIKLF LANEYYKEAL
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Rig interacts with *Drosophila* nuclear receptors

The lethal phenotypes of *rig* mutants, effects on ecdysone-regulated gene expression, and protein interaction domains in the Rig protein sequence raise the possibility that it might act as a nuclear receptor cofactor. Accordingly, we conducted glutathione S-transferase (GST) pull-down assays to determine whether Rig is capable of interacting with

Fig. 4. *rig* encodes a protein with multiple WD-40 repeats and an LXXLL motif. The nine WD-40 repeats identified by at least two of the three protein motif search engines used to analyze the Rig sequence are highlighted in yellow. The LXXLL motif that is associated with known nuclear receptor cofactors is boxed.

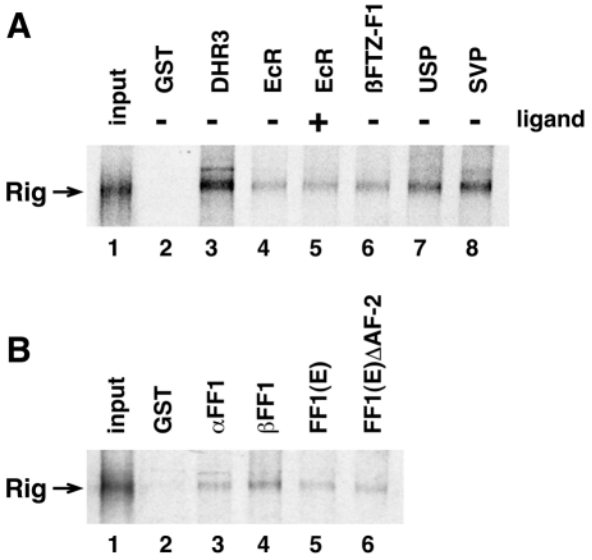

Fig. 5. (A) Rig protein binds to DHR3, EcR, βFTZ-F1, USP and SVP in vitro. In vitro synthesized ^{35}S-labelled Rig was incubated in a batch assay with control GST alone (lane 2) or GST fusions to the indicated receptors in either the presence (+) or absence (–) of ligand (1 μM muristerone A, a potent activator of EcR). Bound Rig was resolved by SDS-PAGE and visualized by autoradiography. Lane 1 depicts 1/5 the amount of input Rig protein, marked by an arrow. (B) Rig binds in vitro to the FTZ-F1 ligand binding domain (LBD) in an AF-2-independent manner. In vitro synthesized ^{35}S-labelled Rig was incubated in a batch assay with control GST alone (lane 2) or GST fusions to full-length αFTZ-F1 (lane 3), full-length βFTZ-F1 (lane 4), the βFTZ-F1 LBD (E region, lane 5) or a mutated βFTZ-F1 LBD in which the core of the AF-2 activation domain was deleted (ΔAF-2, lane 6). Bound Rig was resolved by SDS-PAGE and visualized by autoradiography. Lane 1 depicts 1/5 the amount of input Rig protein, marked by an arrow.

Drosophila nuclear receptors. GST-receptor fusion proteins were expressed in *E. coli*, purified, immobilized on glutathione-Sepharose beads and mixed with in vitro synthesized ^{35}S-labelled Rig. After extensive washing, associated radiolabeled protein was eluted and visualized by SDS-PAGE and autoradiography. As shown in Fig. 5A, Rig binds to GST-DHR3 (lane 3), GST-EcR (lane 4), GST-βFTZ-F1 (lane 6), GST-USP (lane 7) and GST-SVP (lane 8) (SVP is the *Drosophila* COUP-TF ortholog, NR2F3), but not to GST alone (lane 2). The preferential binding to DHR3, SVP and USP was reproducible. Rig also shows no interaction with GST fusions to two different regions of the human α4-subunit of the acetylcholine receptor (amino acids 331-431 and 432-518), as expected, supporting the specificity of its interaction with the *Drosophila* nuclear receptors (data not shown). Rig binding to GST-EcR is unaffected by the addition of ligand (Fig. 5A, lane 5). Similarly, its binding to GST-USP is unaffected by the addition of ligand and/or its EcR heterodimer partner (data not shown). Rig can bind to both the early (α) and late (β) forms of FTZ-F1, which share an identical C-terminal LBD (Lavorgna et al., 1993) (Fig. 5B, lanes 3,4) as well as the FTZ-F1 E region that spans the LBD (amino acids 555-802) (Fig. 5B, lane 5). Interestingly, Rig can also bind to a mutated FTZ-F1 E region that is missing the conserved activation domain 2 core motif located between amino acids 791-797 (Beckstead et al., 2001) (Fig. 5B, lane 6), indicating that it can interact with nuclear receptors in an AF-2-independent manner.

As a test for functional interactions between *Drosophila* nuclear receptors and Rig, we looked for dominant genetic interactions between our *rig* mutations and mutations in *EcR*, *usp*, *βFTZ-F1* or *E75*, as well as recessive genetic interactions with *EcR* or *βFTZ-F1* mutations. Although this study did not reveal a significant genetic interaction (data not shown), the results do not rule out the possibility that Rig interacts functionally with these receptors, as genetic interactions are known to be allele specific. The isolation of additional *rig* alleles using other mutagens, such as EMS, should provide weaker mutations that may prove more useful for genetic interaction studies.

Rig protein shows dynamic subcellular localization in larval cells

Antibodies raised against the C-terminal region of Rig were used to stain organs dissected from staged larvae and prepupae. Rig was detected in the brain (Fig. 6D-F) and salivary glands

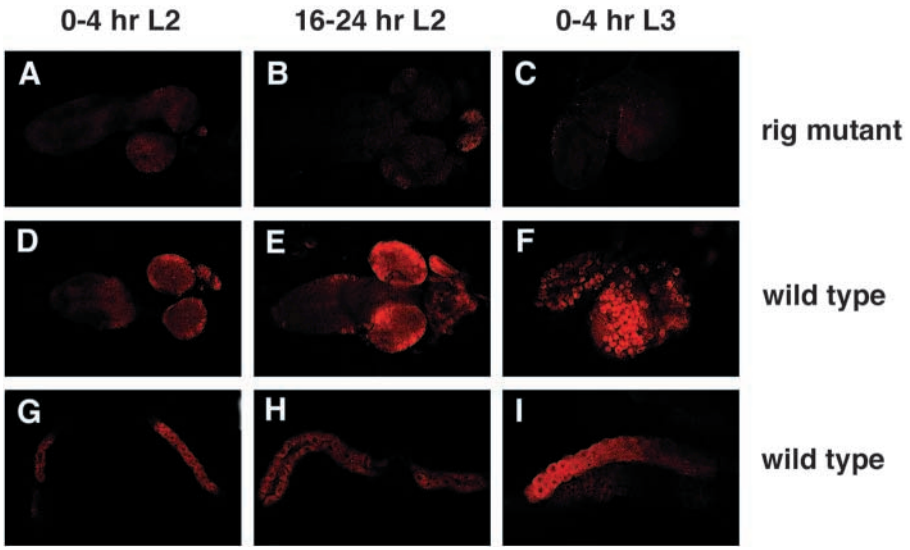

Fig. 6. Rig protein is expressed in the brains and salivary glands of second and early third instar larvae. Organs were dissected from *rig* mutant (*l(2)k07839/Df(2R)exu*^{1}) (A-C) or control (*l(2)k07839/CyO* or *Df(2R)exu*^{1}*/CyO*) (D-I) larvae staged at either 0-4 hours (A,D,G) or 16-24 hours (B,E,H) after the first-to-second instar larval molt, or 0-4 hours after the second-to-third instar larval molt (C,F,I). Brains (A-F) or salivary glands (G-I) were fixed and stained to detect Rig protein. The specificity of the antibodies was demonstrated by the absence of staining in *rig* mutant larvae (compare A-C with D-F). Background staining can occasionally be seen in the ring gland (B).

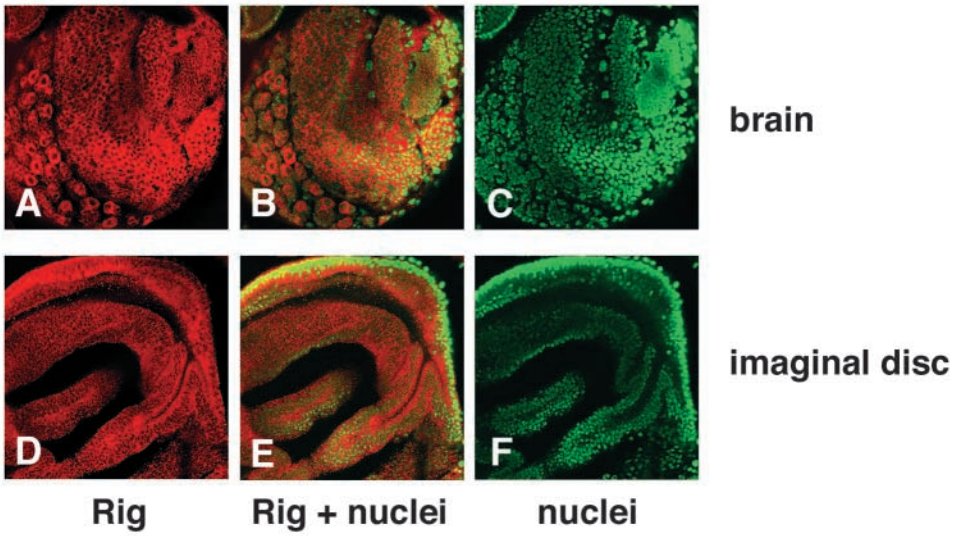

Fig. 7. Rig protein is present in the cytoplasm of brain and imaginal disc cells. Brain complexes (A-C) and wing imaginal discs (D-F) dissected from late third instar larvae were fixed and stained to detect Rig protein (red). A mouse anti-histone antibody was used to counterstain the nuclei (green).

(Fig. 6G-I) of early and late second instar larvae, consistent with the presence of *rig* mRNA at these stages (Fig. 3) and the known essential roles for *rig* during larval development (Table 1). No Rig was detected in tissues isolated from *l(2)k07839/Df(2R)exu*^{1} mutant larvae, indicating that the antibody is specific for Rig protein (Fig. 6A-C). At all stages examined during second and third instar larval development, Rig protein appears to be restricted primarily to the cytoplasm of cells in the brain and imaginal discs – tissues that are fated to form specific parts of the adult fly during metamorphosis (Fig. 7). Rig is also localized to the cytoplasm of larval salivary gland cells during the second (Fig. 6) and early third instar (Fig. 8A-C) stages. Rig protein, however, begins to shift into the nucleus of these cells in the mid-third instar (24-30 hours after the molt, Fig. 8D-F) maintaining this localization through the end of larval development (36-42 hours after the molt, Fig. 8G-I). At puparium formation, Rig protein shuttles out of the nucleus of salivary gland cells to become more abundant in the cytoplasm (Fig. 8J-L). A similar dynamic movement of Rig can be seen in cells of the larval midgut during third instar development. Rig protein is relatively abundant in imaginal cells of the larval midgut, and remains restricted to the cytoplasm (Fig. 9, arrows), similar to its cytoplasmic localization in other imaginal cells (Fig. 7). In contrast, Rig localization within the larval midgut cells is more dynamic. Rig is primarily excluded from the nucleus of larval midgut cells in the early third instar (12-18 hours after the molt, Fig. 9A-C), is approximately equally distributed between the nucleus

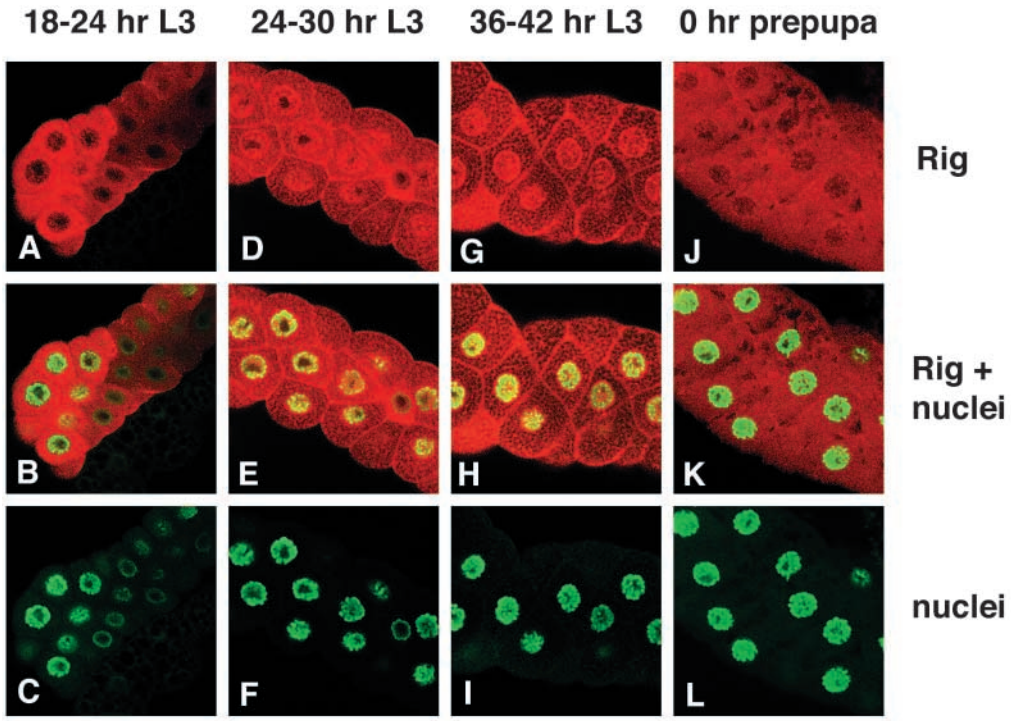

Fig. 8. Rig protein shuttles between the nucleus and cytoplasm in larval salivary gland cells. Larval salivary glands dissected from third instar larvae staged 18-24 hours (A-C), 24-30 hours (D-F), or 36-42 hours (G-I) after the molt, or newly formed prepupae (J-L), were fixed and stained to detect Rig protein (red). A mouse anti-histone antibody was used to counterstain nuclei (green).

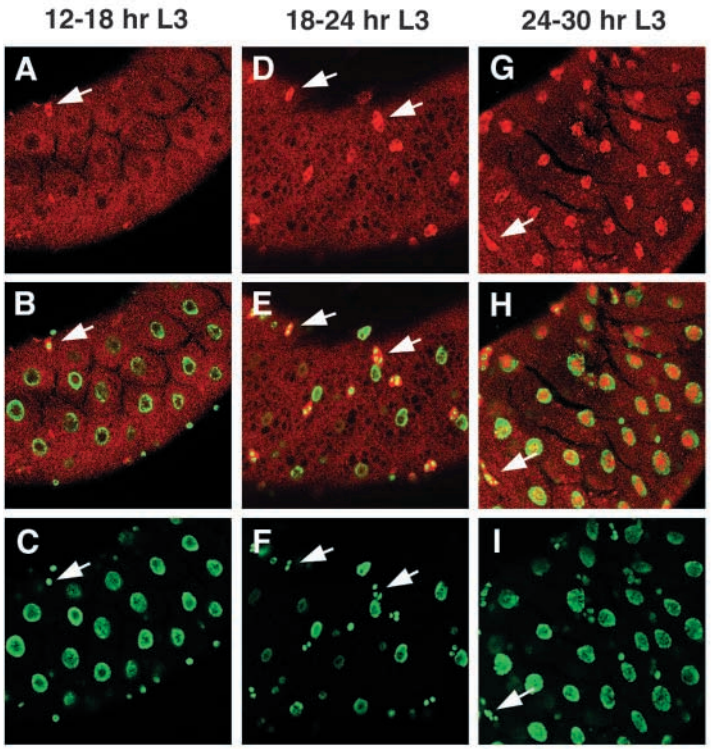

Fig. 9. Rig protein translocates from the cytoplasm to nucleus in larval midgut cells. Larval midguts dissected from third instar larvae staged 12-18 hours (A-C), 18-24 hours (D-F), or 24-30 hours (G-I) after the molt were fixed and stained to detect Rig protein (red). A mouse anti-histone antibody was used to counterstain nuclei (green). Arrows point to clusters of imaginal gut cells.

and cytoplasm of larval midgut cells at 18-24 hours after the molt (Fig. 9D-F) and is nuclear in the larval cells by 24-30 hours after the molt (Fig. 9G-I). Unlike the larval salivary gland expression pattern, however, Rig shuttling into the nucleus of larval midgut cells is not uniform throughout the tissue and thus appears to be spatially regulated. Nuclear localization was seen within distinct clusters of adjacent midgut cells, although it was difficult to determine whether this localization occurred in the same region of the midgut in other animals at that stage (data not shown).

Discussion

rig encodes a novel nuclear receptor interacting protein

Mutations in *rig* result in prolonged second and third instar larval stages, defects in molting, larval lethality and duplicated mouth parts (Table 1, Figs 1, 2). These phenotypes are characteristic of defects in ecdysone signaling, suggesting a critical role for *rig* in ecdysone responses during larval development. Two classes of genes produce mutant phenotypes that resemble those seen in *rig* mutant animals: those required for ecdysone biosynthesis or release – including *ecdysoneless* (*ecd*) (Garen et al., 1977), *dare* (Freeman et al., 1999) and *itpr* (Venkatesh and Hasan, 1997) – and those encoding nuclear receptors that mediate the ecdysone signal – *EcR* (Bender et al., 1997; Schubiger et al., 1998; Li and Bender, 2000), *usp* (Perrimon et al., 1985; Oro et al., 1992; Hall and Thummel, 1998), *E75A* (Bialecki et al., 2002), and *βFTZ-F1* (Yamada et al., 2000). Unlike ecdysone-deficient mutants, the lethal phenotypes of *rig* mutants cannot be rescued by feeding 20E (Table 2), indicating that ecdysone is not limiting in these animals and that *rig* acts downstream from hormone biosynthesis or release. Rather, we propose that Rig is functioning as a nuclear receptor cofactor, based on five lines of evidence. First, the lethal phenotypes of *rig* mutants are very similar to those defined for *EcR*, *usp*, *E75A* and *βFTZ-F1*, although all of these nuclear receptor genes are expressed in an essentially normal manner in *rig* mutant larvae (Fig. 3). Second, *rig* mutants display a defect in the ecdysone-triggered switch in *E74* isoform expression that is characteristic of reduced ecdysone signaling, indicating that *rig* is required for the appropriate expression of specific ecdysone-inducible genes (Fig. 3). Third, these effects on gene expression are likely to be indirect as the predicted Rig protein sequence contains multiple protein-protein interaction domains and no known DNA-binding motifs. Fourth, Rig protein can interact physically with several *Drosophila* nuclear receptors, including EcR, USP and βFTZ-F1 (Fig. 5), all of which have mutant phenotypes in common with *rig* mutants. Finally, as we discuss in more detail below, Rig protein shuttles between the cytoplasm and nucleus of larval cells in a manner similar to the active subcellular redistribution that has been reported for known *Drosophila* and vertebrate nuclear receptor cofactors.

Five *Drosophila* nuclear receptor cofactors have been identified to date: Alien (Dressel et al., 1999), SMRTER (Tsai et al., 1999), MBF1 (Takemaru et al., 1997), Taiman (Bai et al., 2000) and Bonus (Beckstead et al., 2001). Of these, only *bonus* appears to have activities in common with *rig*, although relatively limited genetic studies have been undertaken for most of these cofactors. No mutants have been characterized for SMRTER or Alien, which act as co-repressors in tissue culture transfection assays (Dressel et al., 1999; Tsai et al., 1999). MBF1 null mutants are viable and display a strong genetic interaction with *tdf*/*apontic* mutants that indicate a role in tracheal and nervous system development (Liu et al., 2003). Somatic clones of *taiman* mutants reveal a role in border cell migration during oogenesis (Bai et al., 2000). In contrast, *bonus* mutants display first instar larval lethality as well as defects in salivary gland cell death and cuticle and bristle development, implicating a role for *bonus* in ecdysone responses during development (Beckstead et al., 2001). Also like *rig*, *bonus* mutations result in gene-specific defects in ecdysone-regulated transcription, and Bonus protein can interact with a range of *Drosophila* nuclear receptors, including EcR, USP, SVP, DHR3 and FTZ-F1. Bonus, however, interacts with these receptors in an AF-2-dependent manner, unlike Rig (Fig. 5). Moreover, the larval lethal phenotypes of *rig* mutants do not resemble those reported for *bonus* mutants and, unlike Rig, Bonus protein appears to be exclusively nuclear in both

larval and imaginal tissues. Further work is required to determine whether *bonus* and *rig* might act together to regulate ecdysone response pathways.

Rig is distinct from all known *Drosophila* nuclear receptor cofactors in that it is not part of an evolutionarily conserved protein family. Alien, SMRTER, MBF1, Taiman and Bonus all have vertebrate homologs, and Taiman and Bonus are the fly orthologs of the well characterized vertebrate nuclear receptor cofactors AIB1 and TIF1, respectively (Bai et al., 2000; Beckstead et al., 2001). In contrast, Rig does not contain identifiable enzymatic activities nor the conserved functional domains that define most nuclear receptor cofactors. BLAST searches with the Rig protein sequence did not reveal any closely related sequences in other organisms, although the top hits, which show limited homology in the WD-40 repeats (25-33% identity), are in factors known to modify chromatin, including human histone acetyltransferase type B subunit 2 (RBBP-7) and chromatin assembly factor 1 (CAF-1).

The WD-40 repeats that comprise about half of the Rig protein sequence are likely to play an important role in its activity. Consistent with this proposal, an N-terminal fragment of Rig, containing two WD-40 repeats but missing the LXXLL motif (amino acids 1-300), is capable of interacting with GST-DHR3 and GST-USP, suggesting that these repeats are sufficient for Rig-nuclear receptor interactions (data not shown). WD-40 repeats provide multiple surfaces for protein-protein interactions and have been identified in over 150 proteins that function in a wide range of processes, including cytoskeleton assembly, transcriptional regulation, and pre-mRNA processing (reviewed by Smith et al., 1999). In *Drosophila*, WD-40 repeats are associated with several transcriptional regulators, including the p85 subunit of TFIID (Kokubo et al., 1993), the Polycomb group protein encoded by *extra sex combs* (Gutjahr et al., 1995), and the Groucho corepressor (Stifani et al., 1992). In addition, a WD-40 repeat protein, TBL1, has been identified as part of a multiprotein complex with thyroid hormone receptor that contains the SMRT nuclear receptor corepressor and HDAC-3 (Li et al., 2000). The presence of these sequences in Rig may thus provide a scaffold for protein-protein interactions that could mediate the formation of multiprotein transcriptional complexes on ecdysone-regulated promoters. Further biochemical studies of Rig should provide insights into the significance of its WD-40 repeats as well as a foundation for understanding how Rig exerts its effects on transcription.

Rig may contribute to the spatial and temporal control of ecdysone signaling through its subcellular localization

It is not clear how Rig expression in the brain, imaginal discs and salivary glands of second and third instar larvae is related to the lethal phenotypes of *rig* mutants, although neuroendocrine signaling is clearly required for molting, a process that is defective in *rig* mutant larvae (Riddiford, 1993). The subcellular localization of Rig protein at later stages, however, correlates with the distinct fates of larval and imaginal cells during metamorphosis. Rig protein appears to be restricted to the cytoplasm of cells that are fated to form parts of the adult fly, including neuroblasts, imaginal discs, and the imaginal islands of the larval midgut (Figs 7, 8). In contrast, Rig shows dynamic changes in its subcellular distribution in larval salivary gland and midgut cells, both of which undergo steroid-triggered programmed cell death during metamorphosis. It is possible that these differences in subcellular localization could contribute to the distinct fates of these tissues in response to ecdysone signaling.

In addition to this spatial correlation, there is also a temporal correlation between the times at which Rig protein shuttles between the cytoplasm and nucleus in larval tissues and the coordinated changes in ecdysone-regulated gene expression that occur during the third instar. The switch from cytoplasmic to nuclear localization in larval salivary glands and midguts occurs at approximately the same time, 24-30 hours after the second-to-third instar larval molt (Figs 8, 9), suggesting that Rig may be responding to a common temporal signal. Cell type-specific factors, however, must also contribute to this regulation as Rig is localized to the nucleus of only a subset of cells in the larval midgut (data not shown). Interestingly, this protein redistribution correlates with a poorly understood event that is represented by widespread changes in ecdysone-regulated gene expression, called the ‘mid-third instar transition’ (Andres and Cherbas, 1992; Andres et al., 1993). It is possible that the cytoplasmic-to-nuclear transport of Rig in larval tissues contributes to the regulation of this response, which prepares the animal for metamorphosis one day later. Similarly, Rig returns to the cytoplasm of salivary gland cells at puparium formation, in synchrony with the widespread changes in ecdysone-regulated gene expression associated with the onset of metamorphosis. This translocation, however, is not seen in the larval midgut, where Rig protein remains in the nucleus of some cells (data not shown). Rig shuttling thus appears to be differentially controlled in both a temporally and spatially restricted manner, correlating with major switches in ecdysone-regulated transcription. The observation that the first of these shifts in subcellular distribution occurs during the major lethal phase of *rig* mutants – the mid-third instar (Table 1) – suggests that these intracellular movements contribute to the critical functions of Rig during development.

Interestingly, several recent reports have described the subcellular redistribution of nuclear receptor cofactors in both vertebrate and *Drosophila* cells. The p/CIP vertebrate nuclear receptor coactivator is differentially distributed within the cells of the mouse female reproductive organs (Qutob et al., 2002). For example, p/CIP is detected primarily in the nuclei of highly proliferative follicular cells while it is most abundant in the cytoplasm of terminally differentiated cells of the corpus luteum. p/CIP displays active nucleocytoplasmic shuttling in response to growth factors in cell culture, and interacts directly with the microtubule network in the cytoplasm. Similarly, MEK-1 kinase-mediated phosphorylation of the SMRT mammalian corepressor leads to the translocation of this factor from the nucleus to the cytoplasm in cell culture transfection assays (Hong and Privalsky, 2000). The functional homolog of this protein in flies, SMRTER, also shows active redistribution from the nucleus to the cytoplasm in response to a MAP kinase pathway, in this case mediated by EGFR/Sno/Ebi in the *Drosophila* eye (Tsuda et al., 2002). In both of these systems, regulated phosphorylation of SMRT/SMRTER results in dissociation of a repressor complex and derepression of target gene transcription.

These observations raise the possibility that the subcellular location of Rig could determine its regulatory function in

different cell types. For example, by analogy with SMRT/SMRTER, loss of Rig from the nucleus of larval cells might disrupt a corepressor complex on specific promoters, leading to coordinate target gene derepression. This is consistent with the proposal that the ecdysone receptor exerts critical repressive functions during larval development (Tsai et al., 1999; Schubiger and Truman, 2000). Alternatively, Rig protein in the cytoplasm may tether one or more nuclear receptors, preventing them from acting on their cognate target genes in the nucleus. We do not favor this model, however, because antibody stains reveal an exclusively nuclear localization for EcR, USP and βFTZ-F1 at the onset of metamorphosis (Talbot et al., 1993; Henrich et al., 1994; Yamada et al., 2000). It is also interesting to note that Rig protein appears to localize to discrete regions within the nuclei of larval midgut cells that do not contain chromosomes (Fig. 9G-I) while Rig co-localizes with the giant polytene chromosomes in larval salivary gland cells (Fig. 8D-F). Rig may thus exert some functions in the nucleus that are independent of chromatin binding. Further biochemical studies of Rig, including the identification of additional proteins that interact with this factor, should provide insights into the significance of the subcellular localization of Rig protein as well as a mechanistic understanding of how Rig contributes to ecdysone responses during *Drosophila* larval development.

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