Ecdysone signaling is required for proper organization and fluid secretion of stellate cells in the Malpighian tubules of Drosophila melanogaster

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ABSTRACT Drosophila development is a tightly regulated process involving metamorphosis of a relatively less mobile larva to a highly motile adult, triggered by secretion of 20-hydroxyecdysone. Under the influence of ecdysone, most of the larval tissues degenerate, while the imaginal cells differentiate and form adult specific structures. Although the larval Malpighian tubules do not seem to be affected by ecdysone during metamorphosis, we show that ecdysone signaling plays an important role in the early development and functioning of Malpighian tubules. Disruption of ecdysone receptor function, using targeted expression of dominant negative ecdysone receptor in stellate cells, results in disruption of organization of Malpighian tubules. The number of stellate cells is reduced in such Malpighian tubules. Further, they get clustered rather than distributed in their characteristic wild type pattern. We also demonstrate that expression of Drosophila integrin protein (DRIP), an aquaporin responsible for trans-cellular water transport, is also reduced in stellate cells when ecdysone signaling is disrupted. Our results show that of the three ecdysone receptor isoforms, only EcR-B2 rescues these phenotypes. A similar pattern of stellate cell clustering and reduced expression of DRIP is observed in ecd1, a temperature sensitive mutant, under non-permissive conditions. These results suggest that ecdysone signaling is required for proper patterning and functioning of stellate cells and that EcR-B2 may be the primary isoform required for ecdysone signaling in stellate cells.

KEY WORDS: Drosophila, ecdysone receptor, Malpighian tubule

Introduction

The Malpighian tubules (MTs) in Drosophila are simple epithelial structures, which float in the hemocoel and which perform excretory and osmoregulatory functions analogous to vertebrate renal tubules. MTs comprise of two pairs of tubules which do not open to the outside, but are joined to alimentary canal at the junction of endodermal midgut and ectodermal hindgut. On the basis of the reporter gene expression in different enhancer trap lines, each pair of tubules has been divided into six segments, viz., the initial, transitional, main, lower tubule, upper ureter and lower ureter (Sozen et al., 1997). MTs are made up of two main cell types, the Type I or principal cells (PCs) and Type II or stellate cells (SCs) and four different minor cell types (Sozen et al., 1997). Recent studies have shown that the PCs and SCs of MTs originate from different cell lineages, with PCs originating from ectodermal epithelial buds in the hindgut and the SCs from the caudal mesoderm cells which lie above the hindgut in the region from where MTs bud (Denholm et al., 2003). The SCs undergo mesenchymal to epithelial transformation during intercalation.
and adopt epithelial characteristic once they are incorporated into the tubules and develop an apicobasal polarity. The primary function of MTs is secretion of isotonic fluid, which is accomplished by concerted action of physiologically distinct PCs and SCs. PCs are enriched with vacuolar H⁺-ATPase transporter which use Na⁺/H⁺ and K⁺/H⁺ exchanger to transport cations into the lumen (Davies et al., 1996; Dow et al., 1994, 1998; O’Donnell et al., 1996; Sozen et al., 1997) while SCs express channels which permit flow of Cl⁻ ions (O’Donnell et al., 1998) and aquaporins which permit water flow into the lumen (Dow et al., 1995; Kaufmann et al., 2005). One of the Drosophila aquaporins, Drosophila Integral Protein (DRIP), a water specific aquaporin with very high transport rate is expressed in SCs of MTs (Kaufmann et al., 2005). Expression of DRIP is significantly high during embryonic and adult stages in SCs, which suggests that water moves transcellularly through them (Kaufmann et al., 2005).

MTs are unique in not undergoing extensive morphological changes during metamorphosis from larva to adult. Drosophila metamorphosis, which is coordinated by 20-hydroxyecdysone (referred to as ecdysone) hormone and is characterized by proliferation and differentiation of imaginal cells into adult structures (Davis et al., 2005). One of the isoforms involved. It is still not clear how the MTs remain more or less refractory to ecdysone and are carried over to the adults in knowing the role of ecdysone in incorporation and function of SCs. We expressed a dominant negative mutant EcR (EcR-DN) (Cherbas et al., 2003) using the UAS-GAL4 system (Brand and Perrimon., 1993) in SCs of Malpighian tubules to block ecdysone signaling. Blocking ecdysone signaling using two GAL4 lines, c649 and c724 results in developmental arrest at 1st/2nd instar transition and 1st instar, respectively. We show that the number of SCs is reduced in these mutants. We also show that the intercalation of SCs into the MTs during elongation is disrupted as these larvae show clusters of SCs, unlike the regular arrangement in wild type. Expression of Drosophila aquaporin, DRIP, is also reduced in these SCs. These results were confirmed using the ecd4 temperature sensitive hypomorph allele, (Garen et al., 1977). In this case too, the SCs were irregularly distributed, with a reduced expression of DRIP. Co-expression of only EcR-B2 isoform with the EcR-DN in developing MTs, but not of EcR-B1 or EcR-A isoform, was able to rescue the lethal phenotype suggesting that EcR-B2 may be essential for SCs. A role of ecdysone in SC morphogenesis in MTs is further indicated by the fact that expression of EcR-DN with c724 Gal4 driver which expresses in all the stellate cells results in a more drastic phenotype than with the c649 Gal4 driver which expresses only in a subset of stellate cells.

Results

Disruption of ecdysone receptor results in morphological changes in Malpighian tubules

To examine the role of ecdysone in development of MTs, we used two dominant negative EcR proteins, EcRF645A and EcRW650A, carrying mutations in the ligand binding domain of EcR and thus affecting all isoforms of ecdysone receptors (Cherbas et al., 2003).EcRF645A binds to ecdysone, while EcRW650A does not, although both form heterodimers with USP and bind to the ecdysone response elements. Since they are not able to activate the target gene expression normally, the ecdysone signaling is interrupted when any one of them is expressed (Cherbas et al.,

### TABLE 1

**EXPRESSION DOMAINS OF GAL4 DRIVERS USED IN THE PRESENT STUDY**

<table>
<thead>
<tr>
<th>GAL4 Driver Domains of expression</th>
<th>MUSCULATURE CELLS</th>
<th>ANTERIOR SPIRACLES</th>
<th>ANAL PLATE</th>
<th>VISUAL MUSCULATURE CELLS</th>
<th>STELLATE CELLS</th>
<th>BAR SHAPED STELLATE CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>c649</td>
<td>Bar- shaped stellate cell, salivary glands, proventriculus, mid gut, visceral musculature cells</td>
<td>anterior spiracles, anal plate</td>
<td>visceral musculature cells</td>
<td></td>
<td>Stellate cells</td>
<td></td>
</tr>
<tr>
<td>c724</td>
<td>Stellate cells, bar shaped stellate cells, salivary glands, visceral musculature cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 2

**MEAN DIAMETER OF MALPIGHIAN TUBULES IS AFFECTED BY EXPRESSION OF ECR DN TRANSGENES**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mean (+ S.E.) diameter (in mm) of main segment (N=30 in each case)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>98.0 ± 2.5</td>
</tr>
<tr>
<td>c649 X EcR DN</td>
<td>80.0 ± 2.5</td>
</tr>
<tr>
<td>c724 X EcR DN</td>
<td>50.1 ± 2.0</td>
</tr>
<tr>
<td>c649 X EcR DN, EcR B2</td>
<td>95.2 ± 2.0</td>
</tr>
<tr>
<td>c724 X EcR DN, EcR B2</td>
<td>93.0 ± 2.8</td>
</tr>
</tbody>
</table>

* indicate significant differences in diameter of tubule (P<0.05).
Since EcR<sup>F645A</sup> and EcR<sup>W650A</sup> exhibited identical results, we used EcR<sup>F645A</sup> in the present study and refer to it as EcR-DN in the following text. The EcR-DN was expressed in SCs of MTs with c649 and c724 Gal4 drivers. To identify the different tissues in which these Gal4 drivers express, we used UAS GFP as reporter and observed the expression pattern (see supplementary Fig. S1). It was seen that besides the SCs, these drivers also express in a few other tissues (see Table 1). As shown in Table 1, only the SCs, visceral musculature and salivary glands are common tissues in which both these Gal4 drivers express. Expressing EcR-DN with c649 and c724 Gal4 drivers resulted in slow development and larval lethality. The c649/+; EcR-DN/+ (Fig. 1B) and c724/EcR-DN (Fig. 1C) larvae appeared smaller in size when compared to wild type larvae of similar age (Fig 1A). We looked at the MT as these drivers also express in SCs, we observed that the size of MTs from dying/dead larvae of c649/+; EcR-DN/+ (Fig. 1B; blue arrow and Fig. 1b; black arrows) or c724/EcR-DN (Fig. 1C; blue arrow and Fig 1c; black arrows) was smaller when compared to wild type (Fig.1A; blue arrow and Fig.1a; black arrows). We measured the mean diameter of MTs (Table 2) from wild type across the main segment, which was 98.0 ±2.5 μm, while that in the c649/+; EcR-DN/+ and c724/EcR-DN larvae was 80.0 ±2.5 μm and 50.1 ±2 μm, respectively. Larvae from c724/EcR-DN showed greater reduction in size than c649/+; EcR-DN+. Larvae from these crosses also exhibited other gross abnormalities, like irregular tracheae (Fig. 1B and 1C; red arrow), low adipose tissue, and sluggish movements. Cuticular preparations of dying/dead larvae in these crosses showed defects in larval molting. The cuticular preparation of c649/+; EcR-DN+/+ larvae (Fig. 2B) revealed the presence of both 1<sup>st</sup> (Fig. 2B; black arrows) and 2<sup>nd</sup> (Fig. 2B; red arrow) instar larval mouth hooks, suggesting that these larvae retain the cuticle from previous instar in addition to their newly formed cuticle while the control wild type larvae had already reached 3<sup>rd</sup> instar stage as revealed by the mouth hooks (Fig 2A; black arrow). Cuticular preparations from c724/EcR-DN+; EcR-DN+ larva (Fig. 2C; black arrows), on the other hand, showed mouth hooks characteristic of first instar stage suggesting their death during 1<sup>st</sup> instar itself.

**Disruption of ecdysone receptor leads to reduction in the number of stellate cells**

A remarkable feature of MT is that the number of PCs and the SCs remain fairly constant throughout development (Wessing and Eichelberg, 1978; Sozen et al., 1997). Since following expression of EcR-DN, the MTs were smaller in size, we counted the number of different cell types in MTs from wild type, c649/+; EcR-DN+ and c724/EcR-DN larvae. For an unambiguous identification of the SCs, the MTs were immuno-stained with teashirt antibody, which is a specific marker for SCs (Jung et al., 2005; Singh et al., 2007). Data in Table 3 show that the numbers of PCs in the anterior and posterior tubules of c649/+; EcR-DN+ and c724/EcR-DN genotypes were similar to that in the wild type. However, while the mean numbers of SCs in the wild type anterior and posterior tubules were 31.8 ±0.2 and 21.4 ±0.1, respectively, those in anterior and posterior tubules of c649/+; EcR-DN+ larvae were 24.9 ±0.4 and 17.5 ±0.2, respectively and in c724/EcR-DN MTs, these were 18.5 ±0.6 and 13.7 ±0.4, respectively.
tively. We subjected this data to one way anova followed by post hoc test and found that the number of SCs in \(c_{649}^+/EcR-DN+/\) and \(c_{724}/EcR-DN\) was significantly reduced when compared to wild type \((p<0.05)\). It was also seen that the \(c_{724}\) Gal4 driver caused greater reduction in numbers of SCs than the \(c_{649}\) Gal4 driver (Table 3).

Disruption of ecdysone signaling leads to aberrant pattern of integration of stellate cells

\(Drosophila\) MTs are derived from the ectodermal epithelial buds and the mesenchymal mesodermal tissues (Denholm et al., 2003). During embryogenesis, the principal cells are formed by stage 13 (Skaer, 1989). The SCs are found near the vicinity of MTs at stage 11/12 and at stage 13 the SCs have just started intercalation, but only by stage 15 the SCs are fully incorporated in the MTs (Denholm et al., 2003). SCs are distributed evenly throughout the anterior and posterior tubules at regular intervals (Sozen et al., 1997). Ecdysone plays an important role in embryonic morphogenesis. Maternally deposited ecdysteroids are the source of active ecdysone (Hoffman et al., 1985), however till stage 11 no ecdysone activity is observed. Ecdysone activity is first observed at stage 12 and peaks at stage 13 and continues to be high after that (Kozlova and Thummel, 2003). Since ecdysone activity is first observed just prior to SCs intercalation, it is very likely that this process is governed by ecdysone. We investigated the role of ecdysone in arrangement of the SCs. In wild type larvae (Fig. 3A), the SCs were distributed in the characteristic pattern as reported earlier (Sozen et al., 1997). However, in larvae expressing EcR-DN under the \(c_{649}\) (Fig. 3B) and the \(c_{724}\) Gal4 driver, the regular arrangement of SCs in MTs was lost, instead they were found to be present in clusters suggesting that the intercalation of SCs into the MTs during embryogenesis was disrupted. We counted the number of SC clusters in wild type and compared them with EcR-DN mutant expressing MTs (Table 4). It was found that in 25 wild type MTs examined, there were only five instances when SCs were present in clusters, whereas in similar numbers of \(c_{649}^+/EcR-DN+/\) and \(c_{724}/EcR-DN\) MTs, majority of the tubules showed clusters of SC (Table 4). Further, the number of SCs in each cluster in wild type was never more than two, but the clusters in EcR-DN expressing MTs showed

![Fig. 3. Aberrant intercalation of stellate cells. Expression of EcR-DN in SCs using c649(B) and c724(C) drivers leads to clustering of these cells when compared to regular arrangement of wild type (A). Co-expression of EcR-B2 rescues the clustering phenotypes induced by EcR-DN when driven by c649(D) and c724(E) SCs are identified by anti-teashirt antibody (green fluorescence) and chromatin is stained with DAPI (Pseudocolour red). Arrows indicate single stellate cells while encircled area shows clustering of stellate cells. Bar represents 20 μm.](image)

**Table 3**

<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>142.9 ± 0.3</td>
<td>110.0 ± 0.3</td>
<td>31.8 ± 0.2</td>
<td>21.4 ± 0.1</td>
</tr>
<tr>
<td>(c_{649}X) EcR DN</td>
<td>140.0 ± 0.6</td>
<td>104.1 ± 0.8</td>
<td>24.9 ± 0.4*</td>
<td>17.5 ± 0.2</td>
</tr>
<tr>
<td>(c_{649}X) EcR DN,EcR B2</td>
<td>142.4 ± 0.4</td>
<td>106.3 ± 0.5</td>
<td>28.5 ± 0.4</td>
<td>19.8 ± 0.3</td>
</tr>
<tr>
<td>(c_{724}X) EcR DN</td>
<td>141.9 ± 0.4</td>
<td>109.3 ± 0.5</td>
<td>18.5 ± 0.6</td>
<td>13.7 ± 0.4*</td>
</tr>
<tr>
<td>(c_{724}X) EcR DN,EcR B2</td>
<td>141.1 ± 0.5</td>
<td>102.4 ± 0.9</td>
<td>27.5 ± 0.6</td>
<td>20.2 ± 0.2</td>
</tr>
</tbody>
</table>

* shows significantly reduced stellate cells number \((P<0.05)\).

**Table 4**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Anterior tubules (N=25)</th>
<th>Posterior tubule (N=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total No. of Clusters</td>
<td>Range of cells in a cluster</td>
</tr>
<tr>
<td>WT</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>(c_{649}X) EcR DN</td>
<td>128*</td>
<td>2-6*</td>
</tr>
<tr>
<td>(c_{724}X) EcR DN</td>
<td>95*</td>
<td>2-5*</td>
</tr>
<tr>
<td>(c_{649}X) EcR DN, EcR B2</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>(c_{724}X) EcR DN, EcR B2</td>
<td>10</td>
<td>2</td>
</tr>
</tbody>
</table>

N- total number of MTs counted. * shows the significant increase in cluster number and Size \((P<0.05)\).
The B2 ecdysone receptor isoform is required for maintaining normal stellate cell number and integration

The three EcR isoforms are known to be involved in distinct functions depending upon their spatial and temporal expression (Kim et al., 1999; Snug and Robinow., 2000; Truman et al., 1994). It is known that expression of wild type EcR can to a certain extent, rescue phenotypes resulting from EcR-DN expression (Schubiger, 2003; Cherbas et al., 2003). Therefore, we individually co-expressed each of the wild type EcR isoforms (EcR-B1, EcR-B2 or EcR-A) along with EcR-DN to examine possible rescue. To find out if over-expression of EcR-B1, EcR-B2 or EcR-A with any of the two Gal4 drivers by itself had any detectable phenotype, we expressed each of these isoforms in the wild type background. We did not observe any effect on the viability and phenotype following over-expression of any of these EcR isoforms in the target tissues (data not shown). Then we co-expressed each of these isoforms individually with EcR-DN using the two Gal4 drivers as above. It was found that expression of EcR-B2 isoform only was capable of rescuing the lethality caused by EcR-DN. The morphology and pattern of intercalation of SCs in MTs co-expressing EcR-DN and EcR-B2 under the c649 (Fig. 3D) or the c724 (Fig. 3E) drivers was similar to that in wild type. The numbers of SCs in EcR-B2 co-expressing in MTs were restored to almost to the level in wild type (Table 3). Likewise the mean diameter of these MTs was also found to be not significantly different from that in wild type (Table 2). Co-expression of EcR-B1 and EcR-A along with EcR-DN with any of the two Gal4 drivers, however, was not able to suppress the altered MT phenotype (data not shown).

Expression of dominant negative form of the ecdysone receptor reduces DRIP expression in stellate cells

Expression of DRIP, which facilitates water transport (Dow et al., 1995, Kaufmann et al., 2005), has been reported only in the SCs during embryogenesis and in adults (Kaufmann et al., 2005). To investigate if DRIP is expressed in larvae and if ecdysone signaling is required for DRIP expression, we immunostained wild type larval MTs using anti-DRIP antibody. It was seen that as in embryos and adults (Kaufmann et al., 2005), DRIP is also expressed in the larval MTs and the expression is limited to only SCs (Fig. 4A). Expression of DRIP was greatly reduced in SCs following expression of EcR-DN under the c649 (Fig. 4B) or c724/Gal4 driver (Fig. 4C). For exact comparison we measured DRIP intensity using Profile Display Function of Zeiss LSM Meta 510 software which displays the intensity of fluorescence. The fluorescence intensity of DRIP and DAPI is depicted on the Y axis and the marked line in Fig. 4 (A,B,C,D) starting from tail to arrow head and is plotted on the X axis. We found that the intensity of DRIP (Green colour graph) in c649/+; EcR-DN/+ (Fig. 4b) and c724/EcR-DN (Fig. 4c) was much less than wild type (Fig. 4a). Co-expression of EcR-B2 in MTs expressing EcR-DN under c649 (Fig. 4E,e) and c724 (Fig. 4F,f) Gal4 driver restored the DRIP expression to near wild type levels. As noted with MT morphology, co-expression of EcR-B1 or EcR-A did not elevate the expression of DRIP in SCs (data not shown).

Malpighian tubules of larvae homozygous for temperature-sensitive ecd1 mutant allele exhibit reduced stellate cell numbers and defects in stellate cell intercalation at non-permissive temperature

ecd1' (Garen et al., 1977) is a temperature sensitive allele for

![Fig. 4. Drosophila integrin protein (DRIP) levels are reduced in stellate cells when EcR-DN is expressed. Compared to wild type (A), DRIP expression is less in c649/+; EcR-DN/+ (B) and c724/ EcR-DN (C). Coexpression of EcR-B2 restores DRIP expression to nearly wild type levels when driven by c649(D) and c724 (E). DRIP is labeled with FITC (green fluorescence) and chromatin with DAPI (pseudocolour red). The graph below each slide (a,b,c,d,e) represents the Profile display function of LSM 510 Meta which measures the intensity of fluorescence along the line drawn. In Graph green colour shows intensity of DRIP while red shows the DAPI. Bar represents 20 μm in each case. Arrows passing through different cells show DRIP expression only in stellate cells and intensity in these cells is measured by the Profile display function.]
ecdyson production and is unable to synthesize ecdysones when shifted to 29°C. It’s a hypomorph allele showing lethality at non-permissive conditions. In order to confirm if the observed phenotypes following disruption of EcR is because of ecdyson signaling, we grew ecd² homozygous embryos at non-permissive temperature, and examined the arrangement of SCs in MTs from 1st instar larvae. The SCs in these MTs were indeed clustered (Fig. 5A) similar to that seen when EcR-DN is expressed in MTs (Fig. 3). The expression of DRIP in these SCs was also found to be reduced (Fig. 5D) than in wild type (Fig. 4A). The fluorescence intensity profile of DRIP also showed reduced intensity levels (Fig. 5d,f).

Discussion

In Drosophila, developmental events like molting at different larval instars and metamorphosis during pupal period is triggered by the ecdysteroids. Malpighian tubules is an interesting tissue to study developmental biology, because firstly it does not undergo ecdyson induced degeneration (Jiang et al., 1997) and secondly it is made of cells from two different lineages (Denholm et al., 2003) which have physiologically distinct functions (Davies et al., 1996; Dow et al., 1994, 1998; O'Donnell et al., 1996; Sozen et al., 1997; O'Donnell et al., 1998). Though ecdyson signaling in larval MTs does not lead to cell death, we find that disruption of ecdyson signaling using EcR-DN in SCs during early development results in improper morphogenesis of MTs which affects their functioning as reflected in the reduced DRIP expression. These results clearly suggest that ecdyson has an important role in development and physiology of MTs. Earlier studies on the expression of EcR-DN with Eip, GMR, Ser or dpp Gal4 drivers revealed local as well as a global effects resulting in complete blockage of development (Cherbas et al., 2003) suggesting that there is a molting checkpoint. It is likely that localized malfunctioning of EcR in important tissues is sensed by the surveillance system and in cases of irreparable defects, blocks development at the next ecdyson dependent event. Our data suggest that EcR dependent functioning of SCs in MTs could also be one of the critical functions whose malfunctioning can ultimately lead to larval death.

Our present studies show a new role of ecdyson in proper intercalation of SCs. The normal incorporation of SCs depends on the Hibris protein, which is an ortholog of mammalian protein NEPHRIN. Hibris helps to stabilize the intercalation of SCs in MTs (Denholm et al., 2003, Artero et al., 2006). Hibris acts in cell-cell recognition and attraction during embryogenesis (reviewed by Chen and Olson, 2004). We show that ecdyson signaling affects SC arrangement, leading to the possibility that ecdyson may be regulating hibris expression either directly or indirectly. Since disruption of ecdyson signaling in SCs does not affect PC integration our present results further suggest that the arrangement of SCs and PCs are independent of each other. The arrangement of PCs is largely controlled by the product of rols, since in MTs of rols mutants while the PCs were in clusters; the SCs were normally integrated between the PCs (Putz et al., 2005). Our results show that the intercalation requires ecdyson at some stage and that the EcR-B2 isoform may be the major isoform through which ecdyson signaling is transduced. It is notable in this context that migration of border cells in egg chambers requires Ci/EPB transcription factor encoded by slow border cells (sib0) locus and in sibo mutants, border cells migrate either very slowly or not at all (Rorth et al., 1998). Expression of EcR-DN driven by sibo-GAL4in ovaries, results in failure of border cells migration which is subsequently rescued by the expression of EcR-B2 isoform (Cherbas et al., 2003). In view of our present results, it is tempting to speculate that the developmental migration of different cells is triggered by ecdyson and EcR-B2 may be the key isoform that transduces the signal.

SCs play an important role in fluid secretion and inhibition of chloride channels affects functioning of SCs and ultimately affects fluid secretion (Sozen et al., 1997). In hibris mutants, where the number of SCs is reduced, the fluid transport is also affected, resulting in lethality (Denholm et al., 2003). In agreement with some early reports that ecdyson plays a role in fluid secretion by altering membrane permeability (Gee et al., 1977; Ryerse, 1978), we provide direct evidence that ecdyson, directly or indirectly controls the fluid secretion of SCs via its influence on the expression of

Fig. 5. Ecdyson levels affect stellate cell organization and Drosophila integrin protein (DRIP) levels. Immunostaining with anti-teashirt antibody in ecd² mutant shows clusters of SCs (A). DAPI (B) and merged (C). ecd² mutants also show reduced expression of DRIP (D), DAPI(E) and merged(F). The intensity of DRIP(d,f) is measured by Profile display function of LSM Meta 510 software. DAPI is pseudocolour red. Bar represents 50 μm (A,B,C) and 5 μm in (D,E,F).
DRIP.

Stellate cells have an important role and the appropriate number of SCs is essential for proper development. This is evidenced by the fact that the phenotypes derived from c724 are more drastic than similar phenotypes observed by c649. This could be because c724 affects the entire stellate cells, whereas the c649 affects only a small proportion of bar shaped stellate cells.

Our present results thus show that proper ecdysone signaling via the EcR-B2 in MTs during embryonic development is required for correct integration of SCs in developing MTs and for their physiological functions involving DRIP expression.

Materials and Methods

Drosophila stocks

Following fly stocks used in the present study were obtained from the Bloomington stock centre unless mentioned otherwise. Wild type Oregon R*, stellate cells specific GAL4 drivers c724 and c649 (kind gift by Dr. J. A. T. Dow, Institute for Biomedical Sciences, University of Glasgow, UK), UAS responders, P{UAS-EcR.B1-DeltaC655; F645A} (EcR F645A) P{UAS-EcR.B1-DeltaC655; W580A} (EcR W580A) (P{UAS-EcR.B1} (EcR-B1), P{UAS-EcR.B2} (EcR-B2), P{UAS-EcR.A} (EcR-A). Temperature sensitive hypomorph allele for ecdysone (ecf* st red el ca*) (ecf*) (Garen et al., 1977) was obtained from Dr. C. Thummel (Department of Human Genetics, University of Utah School of Medicine, UT). Appropriate crosses were set up to generate P{UAS-EcR.B1-DeltaC655; F645A}; P{UAS-EcR.B1}, P{UAS-EcR.B1-DeltaC655; W580A}; P{UAS-EcR.B1-DeltaC655; F645A}; P{UAS-EcR.B2}; P{UAS-EcR.A} (EcR-A). The temperature sensitive hypomorph allele for ecdysone (ecf*) flies were allowed to lay eggs at 25±1°C and then shifted to 29°C for further development.

Morphological analysis of larvae and Malpighian tubules

Larvae of different genotypes were collected from food plates and washed with distilled water. They were anaesthetized with ether and at 12 hours dark: 12 hours light cycle. For checking lethality, egg laying was set on agar plates and the number of eggs laid was counted. After hatching they were counted and checked for the stage of lethality. In the rescue experiments, similar procedure was done and finally the number of adults emerged was used to calculate the percentage of rescue. The ecf* flies were allowed to lay eggs at 25±1°C and then shifted at 29°C for further development.

Immunocytochemistry

Malpighian tubules from larvae of appropriate genotypes were dissected in 1XPBS, fixed in 4% formaldehyde for 20 min at RT, rinsed in PBST (PBS, 0.1% Triton X-100) and processed for immunostaining essentially as described earlier (Patel NH, 1994). The MTs were mounted in antifadant, DABCO (Sigma). The primary antibodies used were anti-teashirt used at 1:3000 and anti-DRIP used at 1:1000. Goat-anti rabbit AF488 (Molecular probes, USA) was used as secondary antibody. Chro-matin was stained with DAPI (1 μg/ml in 1XPBS). All preparations were analyzed on a Zeiss LSM 510 Meta Confocal microscope and images were processed with Adobe Photoshop.

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References


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