Two-step induction of chordotonal organ precursors in *Drosophila* embryogenesis

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SUMMARY

The chordotonal (Ch) organ, an internal stretch receptor located in the subepidermal layer, is one of the major sensory organs in the peripheral nervous system of *Drosophila melanogaster*. Although the cell lineage of the Ch organ has been well characterized in many studies, the determination machinery of Ch organ precursor cells (COPs) remains largely unresolved. Here we report that the rhomboid (rho) gene and the activity of the Drosophila EGF receptor (DER) signaling pathway are necessary to induce specifically three of the eight COPs in an embryonic abdominal hemisegment. The cell-lineage analysis of COPs using the yeast flpase (flp/FRT) method indicated that each of the eight COPs originated from an individual undifferentiated ectodermal cell. The eight COPs in each abdominal hemisegment seemed to be determined by a two-phase induction: first, five COPs are determined by the action of the proneural gene atonal and neurogenic genes. Subsequently, these five COPs start to express the rho gene, and rho activates the DER-signaling pathway in neighboring cells and induces argos expression. Three of these argos-expressing cells differentiate into the three remaining COPs and they prevent neighboring cells from becoming extra COPs.

Key words: *Drosophila*, sensory organ precursor, chordotonal organ, peripheral nervous system, rhomboid, EGF receptor, argos, atonal

INTRODUCTION

The close regulation of specific numbers of cells plays an important role in the generation of morphological patterns during an animal’s development. A precise and reproducible pattern of sensory organ precursor cells (SOPs) in the peripheral nervous system (PNS) of the fruit fly, *Drosophila melanogaster*, provides a very useful model system to study the mechanism of the regulation of specific cell numbers.

The embryonic PNS includes approximately 650 neurons, and is made up of three major clusters of neurons and support cells located at ventral, lateral and dorsal levels in each segment (Fig. 1A; Campos-Ortega and Hartenstein, 1985). In the PNS of each body segment, two major types of sensory organs can be distinguished by their morphologies. One sensory organ is a chordotonal (Ch) organ composed of subepidermally located internal stretch receptors. The other is an external sensory (Es) organ composed of mechanoreceptors in the cuticle. The determination of Es organ precursors has been well characterized in studies of the development of microchaetes and macrochaetes (Hartenstein and Posakony, 1989). Each Es organ, which is composed of four cells (a neuron and three support cells), is derived from a single Es organ precursor cell (Bodmer et al., 1989; Brewster and Bodmer, 1995, 1996). The determination of Es organ precursors appears to take place in a stepwise fashion (Ghysen and Dambly-Chaudière, 1989). First, subsets of dorsal ectodermal cells are specified to become Es organ precursors by the action of the proneural gene, the *achaete-scute complex* (AS-C) (Campuzano et al., 1985; Skeath and Carroll, 1991). The AS-C apparently controls the expression of genes necessary for the commitment of Es organ precursors. Subsequently, the AS-C expression in the Es organ precursor becomes stronger and the Es organ precursor prevents neighboring cells from adapting a similar developmental fate by lateral inhibition mediated by neurogenic genes such as *Notch* and *Delta* (Lehmann et al., 1983; Dietrich and Campos-Ortega, 1984; Simpson, 1990). Subsequent to its determination, each Es organ precursor follows a stereotyped pattern of division to generate the component cells of each Es organ (Hartenstein and Posakony, 1989). In contrast to the determination of Es organ precursors, that of Ch organ precursors (COPs) remains largely unsolved. Each Ch organ is composed of four cells (a neuron, a scolopale cell, a cap cell and a ligament cell) which are derived from one COP by three successive asymmetric cell divisions (Bodmer et al., 1989; Brewster and Bodmer, 1995, 1996). Each abdominal hemisegment has eight Ch organs (five in LCh5, VChA, VChB and VCh1; Fig. 1A). Although the function of neurogenic genes in determining each SOP seems common to both the Ch organ and the Es organ (Goriely et al., 1991), the removal of the AS-C function does not affect the development of Ch organs (Dambly-Chaudière and...
Ghysen, 1987). Recently, a proneural gene required for Ch organs and photoreceptor cells, atonal (ato), was identified, and its mutant phenotype and expression pattern were examined (Jarman et al., 1993a, 1994, 1995). Loss of ato function results in the elimination of all eight Ch organs, which are normally found in each abdominal hemisegment (Fig. 1B). Although ato regulates the development of all eight Ch organs, only five COPs express ato transcripts in each abdominal hemisegment. The origin of three of the eight Ch organs has remained unknown. The cell lineage of all eight COPs cannot be explained by the machinery used in the Es organ precursor formation. However, Ch organ phenotypes of the following three mutants may help us to resolve the cell lineage of all eight COPs. The spitz (spi) and rhomboid (rho) loss-of-function mutants lack three Ch organs, i.e., two Ch organs in LCh5 and VChA in the ventral cluster, but the remaining five Ch organs are unaffected (Fig. 1C; Bier et al., 1990; Rutledge et al., 1992). The mutant with the loss of argos function forms extra Ch organs (Fig. 1D), and overexpression of argos results in the same phenotype as the spi and rho loss-of-function mutants (Okabe et al., 1996). As Ch organs in these mutants retain their morphology, these genes are required for the determination of a subset of COPs rather than for the regulation of the cell lineage of sibling cells derived from each COP. The spi gene, which encodes a Drosophila homologue of the vertebrate TGF-α, is ubiquitously expressed during embryogenesis (Rutledge et al., 1992), and is a ligand of the Drosophila EGF receptor (DER) (Price et al., 1989; Scheijer and Shilo; 1989; Schweitzer et al., 1995a). The rho gene that encodes a transmembrane protein (Bier et al., 1990) is expressed dynamically and is involved in the regulation of DER-signaling activity in restricted locations (Sturtevant et al., 1993, 1996; Golembio et al., 1996a). The argos gene encodes a secreted molecule that functions as an inhibitor of the signal triggered by the activation of DER signaling (Freeman et al., 1992a; Kretzschmer et al., 1992; Okano et al., 1992; Schweitzer et al., 1995b; Golembio et al., 1996b; Sawamoto et al., 1996). All three genes are involved in the DER-signaling pathway, and functions of these genes have been well characterized in the development of photoreceptor cells (Freeman et al., 1992a,b; Freeman, 1994a,b; Kretzschmer et al., 1992; Okano et al., 1992; Sawamoto et al., 1994, 1996; Tio et al., 1994). Moreover, Bodmer et al. (1989) showed that three COPs were determined subsequent to the other five COPs, based on their BrdU labeling. Based on their positions, the three Ch organs (derived from these three COPs) correspond rather well to the three lost Ch organs in rho loss-of-function mutants.

We have focused on the functions of the rho gene and the DER-signaling pathway in the development of Ch organs. Our results indicate that COPs are determined by two different phases. First, five of the eight COPs are determined by the actions of ato and neurogenic genes. Subsequently, rho gene products in the first five COPs induce the determination of the three remaining COPs, for which DER-signaling activity is required.

MATERIALS AND METHODS

Fly stocks

ato1 was kindly provided by Dr Y. N. Jan, and rhoBdel1 and HS-rho1B were gifts from Dr E. Bier. pnn2.182, pnn4.543A53 and yam2 were obtained from Dr G. M. Rubin. flb2.165 was provided by Dr T. Schüpbach. Ras12F was a gift from Dr Y. Nishida. Act-Draf-lacZ:MKRS/TM2 was provided by Dr K. Kimura. Df3Rip13, red e/TM6B and y w hs-flp:Dr/TM3 were obtained from the Bloomington stock center. To discriminate between heterozygous and homozygous mutant embryos, balancer chromosomes carrying P-elements expressing laclZ (CyO. P[wg-lacZ] and TM3,Sb P[Ubx-lacZ]) were used in all experiments described in this paper. Canton-S was used as the wild-type stock.

rho-overexpression experiment

Canton-S and hs-rho1B embryos were collected on apple agar plates for 2 hours in a moist chamber at 25°C. The embryos were incubated for 4 hours at 25°C, and then placed in a humidified 37°C incubator for 30 minutes. Then the treated embryos were aged at 25°C, collected, fixed and immunostained to assess the number of Ch organs or COPs.

In situ hybridization

Whole-mount in situ hybridization was conducted by using digoxigenin-labeled RNA probes as described by Tautz and Pfeifle (1989). All digoxigenin-labeled RNA probes were prepared using a standard procedure for a digoxigenin RNA-labeling kit (Boehringer Mannheim). ato cDNA (p84F#2; a gift from Dr Y. N. Jan), rho cDNA (a gift from Dr E. Bier) and argos cDNA (cSty-2; Okano et al., 1992) were used for templates.

Immunohistochemistry

Immunohistochemistry was performed as described by Patel (1994) using the following primary antibodies: anti-Couch potato rabbit antiseraum used at 1:5000 dilution, 22C10 mouse monoclonal antibody used at 1:5 dilution and rabbit anti-β-galactosidase polyclonal antibody (Cappel) used at 1:500 dilution. The secondary antibodies were HRP-conjugated goat anti-mouse or rabbit IgG (Jackson Immunoresearch) used at 1:500 dilution.

Double labeling of embryos with antibody and RNA probe

The embryos were collected, dechorionated, and fixed in 4% paraformaldehyde in PBS and devitellinized with methanol. The embryos were rinsed with PBTH (PBS, 0.1% Tween 20, 50 μg/ml heparin, 250 μg/ml tRNA, 1.5 mM DTT) and incubated with anti-Asense rabbit antisera (used at 1:200), diluted in PBTH containing RNasin (Promega). After several washes in PBTH, the embryos were incubated at room temperature for 2 hours in secondary antibody diluted with PBTH containing RNasin. Preparations were washed several times in PBTH, then incubated in a 0.5 mg/ml diaminobenzidine (DAB, Sigma) solution containing 0.03% H2O2 in PBTH. After the color detection, in situ hybridization was performed. We used both primary and secondary antibodies to a 5× higher concentration than is normally used for immunohistochemistry.

Lineage analyses using the yeast flipFRT method

lacZ-expressing clones were generated using the yeast flipFRT site-specific recombination method according to Struhl and Basler (1993). Homozygous hs-flp females were crossed with Act-Draf-nuclacZ males. Fl1 embryos, hemizygous for hs-flp and heterozygous for ActDraf-nuclacZ, were collected on apple agar plates at 18°C and heated to 36°C for 20 minutes, 2-4 hours after egg laying. The embryos were subjected to heat shocks in a water bath in which the water had been prewarmed to 36°C. The embryos were then allowed to develop at 18°C until stage 16. They were subsequently fixed and stained with antibodies.

RESULTS

rho- and ato-expressing COPs

The loss-of-function mutant rho is known to have a loss of...
Fig. 1. The embryonic PNS in an abdominal hemisegment of wild-type and mutant embryos. In red: chordotonal (Ch) organs, in white: external sensory (Es) organs, in gray: multiple dendritic neurons. Nomenclature is according to Brewster and Bodmer (1995). (A) Wild type, (B) atonal mutant, (C) rhomboid mutant and spitz mutant, (D) argos mutant. Extra Ch organs are indicated in black.

Fig. 2. The rho gene is expressed in five ato-expressing COPs in stage 11 embryos. Anterior is to the left and dorsal is to the top. A-C show rho mRNA expression in the wild-type embryo. Soon after the delamination of the first COP, the COP starts to express rho transcripts (arrowheads). Finally five COPs express rho (C). Simultaneously, rho transcripts appear in tracheal pits (indicated by T). (D,E) Double staining with rho antisense RNA probe (blue) and anti-Ase antibody (brown), which labels all SOPs (D), and with the ato antisense RNA probe and anti-Ase antibody (E), respectively. Judging from their relative position to tracheal pits and Ase-positive cells, rho-expressing COPs are identical to ato-expressing COPs (arrowheads). One rho-expressing COP had already turned off the ato expression and started to express the Ase protein (arrow). (F) In ato1/Df(3R)p13 embryos, rho expression is absent in COPs, but its expression in tracheal pits and mesodermal cells remains intact (arrow).

Fig. 3. Ectopic overexpression of the rho gene causes the overproduction of Ch organs. (A,C) Embryos stained with anti-Cpo antibody; (B,D) and tracings of these photographs. Anterior is to the left and dorsal is to the top. (A,B) The lateral clusters of A2-A4 segments in the hs-rho embryo were not subjected to heat shock treatment. Each lateral cluster includes five Ch organs in LCh5. (C,D) The lateral clusters of A2-A4 segments in the hs-rho embryo were subjected to heat shock treatment. Each LCh5 cluster has an extra Ch organ (six Ch organs in each LCh5). The four cells that made up each Ch organ are indicated as follows: c, cap cell; s, scolopale cell; n, neuron; l, ligament cell.
three of the eight Ch organs in each abdominal hemisegment (Figs 1C and 4F; Bier et al., 1990). It was also previously reported that rho expression in COPs corresponded to lacZ-expressing cells in the A18 enhancer trap line (Ghysen and O’Kane, 1989; Bier et al., 1990; Sturtevant et al., 1996). To learn the function of the rho gene during the development of Ch organs, we observed the detailed expression pattern of rho transcripts during Ch organ formation by in situ hybridization. The first rho-expressing cell was observed in the subepidermal layer at stage 10 in the lateral ectoderm of the posterior compartment (Fig. 2A). The number of rho-expressing cells increased stepwise, and five rho-expressing cells were eventually formed in each abdominal hemisegement (Fig. 2C). These five rho-expressing cells were identical to five ato-expressing COPs, based on the relative positions of these five rho-expressing cells and five ato-expressing COPs to Asense (ase) expressing cells (Brand et al., 1993) (Fig. 2D,E). Furthermore, this rho expression in COPs was completely absent in ato null embryos while the rho expression in tracheal primordia was unaffected (Fig. 2F). rho expression continued until the end of stage 11, and rho expression was no longer observed around developing Ch organs after stage 12 (data not shown) when each COP entered into the cell cycle for its progeny formation (Bodmer et al., 1989). As no rho expression was observed in the epidermal layer, we concluded that rho expression started in five ato-expressing COPs after their delamination into the subepidermal layer.

Overexpression of rho caused an increase of Ch organs

rho gene products in five ato-expressing COPs seemed to be necessary to induce the formation of the other three COPs. To confirm this possibility, we overexpressed the rho gene products when the COPs appeared and examined the consequences. The overexpression experiment was carried out using the hs-rho transformant line carrying a full-length rho cDNA under the control of the hsp70 promoter (Sturtevant et al., 1993). The hs-rho embryos subjected to heat-shock showed an increase of one or two Ch organs in LCh5 and in the ventral cluster (Fig. 3C,D and Tables 1 and 2). Based on the staining pattern with anti-Couch potato (Cpo) antibody that labels the nuclei of all four cells in each Ch organ to differing intensities (Bellen et al., 1992), the extra Ch organs retained their morphology, and the cell identities of all four cells in each extra Ch organ were unaffected. The heat-shock treatment had no apparent effect on the pattern of development of Ch and other sensory organs in wild-type embryos (data not shown). We observed no significant change in the number of ato-expressing COPs upon overexpression of rho (data not shown). These results indicated that overexpression of rho was able to induce extra COPs that lacked ato expression.

The DER-signaling cascade was required in the development of the three COPs

The rho gene product induced the formation of the three COPs after the formation of the first five rho-expressing COPs. To confirm whether the phenotype of loss of rho function can be interpreted by the failure of DER-signaling activation, we observed the number of Ch organs in mutant embryos of this signaling pathway. Homozygous embryos of the null mutant

<table>
<thead>
<tr>
<th>Genotype</th>
<th>LCh5</th>
<th>V'Ch1</th>
<th>VChA</th>
<th>VChB</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>hs-rho</td>
<td>5.8</td>
<td>1.3</td>
<td>1.0</td>
<td>1.6</td>
<td>39</td>
</tr>
<tr>
<td>Wild-type</td>
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<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>48</td>
</tr>
</tbody>
</table>

Table 1. The number of chordotonal organs in one abdominal hemisegement

<table>
<thead>
<tr>
<th>Segments with extra Ch organs</th>
<th>n</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCh5</td>
<td>23</td>
<td>59</td>
</tr>
<tr>
<td>V'Ch1</td>
<td>10</td>
<td>26</td>
</tr>
<tr>
<td>VChA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VChB</td>
<td>20</td>
<td>53</td>
</tr>
</tbody>
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Table 2. The number of hemisegments containing extra Ch organs

DER (Fig. 4B; Nüsslein-Volhard et al., 1984), down stream of receptor kinase (Drk) (Simon et al., 1993; data not shown), Son of sevenless (Sos) (Rogge et al., 1991; data not shown), Ras1 (Fig. 4C; Simon et al., 1991) and pointed (pnt; Fig. 4D; O’Neill, et al., 1994) showed a loss of the same three Ch organs in each abdominal hemisegement (VChA and two in LCh5), just as in the rho and spi mutants (Figs 1C, 4F). Cell-type identities in the remaining Ch organs were not affected. However, a loss of negative regulators such as Gap1 (Gaul et al., 1992; data not shown), yan (Fig. 4E; Lai et al., 1992) and argos (Okabe et al, 1996) in this signaling pathway resulted in one or two additional Ch organs in each hemisegement. The level of DER-signaling activity seemed to correspond well to the number of recruited COPs. These results also indicated that the formation of five COPs that developed in V’Ch1, VChB and three in LCh5 does not require DER-signaling activity, while the formation of the remaining three COPs depends on DER-signaling activity.

argos expression in cells surrounding COPs depended on the rho function and DER-signaling activity

While Gap1 and yan gene products function as negative regulators of the DER-signaling pathway intracellularly, Argos, a secreted inhibitor, may act intercellularly to prevent the hyper-activation of the DER-signaling pathway in neighboring cells. We previously reported that argos was expressed in cells surrounding COPs to negatively control the number of Ch organs (Figs 1D, 5A; Okabe et al., 1996). This argos expression in cells surrounding rho-expressing COPs is likely to be regulated by rho in COPs and DER-signaling activity. Therefore, argos expression was examined in the pntP2 null mutant and in the rho null mutant. The pntP2 gene encodes a transcriptional factor containing an ETS domain (Klämbt, 1993) and is considered to be a direct target of MAPK (Biggs et al., 1994) which acts downstream of the DER-signaling pathway during photoreceptor cell development (O’Neill et al., 1994). The presence of pntP2-expressing ectodermal cells at stage 11 was also previously described (Klämbt, 1993). We examined and confirmed that pntP2 is expressed in ectodermal cells surrounding COPs in the posterior compartment in a way similar to argos expression (data not shown). argos transcripts in cells surrounding COPs were eliminated in the rho null mutant (Fig. 5B) and were greatly reduced in the pntP2 null mutant (Fig. 5C). These results indicated that argos expression in cells sur-
ranging rho-expressing COPs depended on the rho function and DER-signaling activity.

Three DER-signaling dependent COPs were selected from argos-expressing cells

As argos expression depended on rho and DER-signaling activity, the DER-signaling pathway should have been strongly activated in these argos-expressing cells. We noted that the loss of rho or pnrP2 function resulted in the elimination of argos expression in a few undifferentiated ectodermal cells that contacted each rho-expressing COP (Fig. 5A). As the differentiation of the three later COPs depended on DER-signaling activity, these argos-expressing undifferentiated ectodermal cells are strong candidates for the progenitors of these three COPs. In order to confirm this possibility, we examined whether some of these argos-expressing cells would start to express a neuronal marker, ase (Brand et al., 1993; Jarman et al., 1993b). We performed double labeling using digoxigenin-labeled argos antisense RNA probe and anti-Ase antibody, and observed embryos carefully between late stage 11 and early stage 12. argos expression in cells surrounding rho-expressing COPs were observed briefly during stage 11, but they disappeared immediately by the end of stage 11. At the end of stage 11, however, we identified two large subepidermal cells that barely continued the expression of argos transcripts in their cytoplasm and weakly expressed Ase proteins in their nuclei (indicated by a black arrow in Fig. 5D; the other double-positive cell is not shown). This argos and Ase double-positive cell was next to an Ase single-positive cell (indicated by a white arrow in Fig. 5D), which seemed to be identical to the rho-expressing COP located in the middle (indicated by 3 in Fig. 2C). The other double-positive cell seemed to be next to the second most ventral COP (indicated by 4 in Fig. 2C; data not shown). We also observed that another argos-expressing cells next to the Ase single-positive cell did not express Ase in its nucleus (indicated by the arrowhead in Fig. 5D). We were not able to identify the last (the third) COP that might also double label for argos and Ase, possibly because of the rapid disappearance of argos expression. However, these results indicated that three COPs (argos-expressing COPs) dependent on DER activity seemed to originate from argos-expressing undifferentiated ectodermal cells surrounding rho-expressing COPs.

Lineage analysis of COPs using the yeast flp/FRT method

As mentioned above, the three argos-expressing COPs did not seem to be derived from the five rho-expressing COPs by cell division. To confirm whether all eight COPs originate from eight individual undifferentiated ectodermal cells, we induced lacZ expression randomly in cells in proneural clusters using the method of site-specific recombination by the yeast flp recombinase (Fig. 6A; Struhl and Basler, 1993; Brewster and Bodmer, 1995, 1996). Small clones of lacZ-positive cells were randomly generated in embryos, and then the embryos were double-labeled with anti-β-galactosidase antibody and the monoclonal antibody 22C10 (Fujita et al., 1982), which labels neuronal cell membranes to help identify Ch organ clones. We examined about 5000 embryos and counted the number of Ch organs in which all four cells were labeled without any lacZ-positive clones in the epidermal cells (except two attachment cells in LCh5 reported by Brewster and Bodmer, 1996) within the same abdominal hemisegment (Fig. 6B). Forty Ch organ clones that satisfied these criteria were obtained. Each clone labeled only one Ch organ, and we never observed more than two labeled Ch organs simultaneously within one hemisegment (Fig. 6C). Therefore, we concluded that each one of the eight COPs is independently derived from one undifferentiated ectodermal cell.

DISCUSSION

Much interest has been paid to the mechanisms of SOP determination in Es organ development and the cell-fate decision of its sibling cells. However, the mechanism of COP determination remains largely unresolved. In this study, we examined the process of COP formation using the rho mutant, several mutants of DER signaling and a cell lineage analysis by the flp/FRT method. Consequently, we showed that the DER-signaling pathway was involved in the determination of three argos-expressing COPs in each abdominal hemisegment. In contrast to the determination of the Es organ precursor, COPs were determined not only by the activities of proneural and neurogenic genes but also by the activity of the DER-signaling pathway.

Each COP is derived from an individual undifferentiated ectodermal cell

Brewster and Bodmer (1995, 1996) previously performed PNS cell lineage analysis using the flp/FRT method. They concluded that the origins of COPs in two ventral Ch organs (VChA and B) and V’Ch1 were individual undifferentiated ectodermal cells. However, the origin of the five Ch organs in LCh5 remained undetermined, because they observed lacZ clones in LCh5 clusters consisting of two or more fully labeled Ch organs in their experiments. They offered the following two possibilities to interpret the pattern of lacZ clones in LCh5. One possibility was that two or more Ch organs within LCh5 were derived from a common precursor cell, and the other possibility was that the labeled Ch organ may have originated from a recombination event in an early ectodermal cell, which, after further cell divisions, gave rise to independent COPs. To demonstrate the latter possibility, we paid attention to the following points in our flp/FRT experiment. We compared two independent hs-flp transformant lines with the most suitable temperature for inducing a small number of lacZ-expressing clones before use. We then chose the hs-flp line that required a higher temperature (36°C) to induce recombination and which showed a lower frequency of recombination (a few clones per embryo), because the frequency of recombination events in the other hs-flp line is so high that it does not seem to be suitable for clonal analyses (recombination can be induced at less than 25°C). Nevertheless, we found two embryos with two labeled Ch organs and a few labeled epidermal cells in one abdominal hemisegment (data not shown). As this finding may be due to an early recombination event prior to the proneural cluster formation in the youngest embryos collected, we did not count the clones that were co-localized with other clones present in the epidermis in the same abdominal hemisegment. On the basis of experiments under these conditions, we observed that almost all Ch organ clones were the only labeled cells in each abdominal hemisegment. Our results indicated that all eight COPs developed from eight individual undifferentiated ectodermal cells. It is not likely
that more than two COPs were derived from a common Ch organ precursor.

**What is the function of rho gene products in five COPs?**

The rho gene expresses a highly localized pattern corresponding to cells that require the activation of the DER-signaling pathway (Raz and Shilo, 1993; Sturtevant et al., 1993, 1996; Golembo et al., 1996a). However, the molecular mechanism underlying the activation of the DER-signaling pathway by Rho protein has not been elucidated. One possibility is that Rho protein interacts with DER protein directly or indirectly to amplify DER signaling intracellularly (Sturtevant et al., 1993). In this hypothesis, Rho proteins should be expressed in cells that require the activation of the DER-signaling pathway. In the development of adult wing veins, rho is expressed in wing vein primordia that require the activation of the DER-signaling pathway for their differentiation (Sturtevant et al., 1993). In another example, rho is expressed in the dorsal follicular cells during oogenesis and is required for amplification of the DER signaling to specify dorsal follicular-cell identity (Rouhola-Baker et al., 1993). The ligand of DER, Gurken, which has a high homology to the mammalian TGF-β, is expressed in the oocyte but not in rho-expressing dorsal follicular cells (González-Reyes et al., 1995). Alternatively, Rho protein is required for the activation of a DER ligand, the Spi transmembrane protein (mSpi), by processing it into the functional soluble form (sSpi; Golembo et al., 1996a). In this hypothesis, Rho protein does not need to be expressed in cells that require DER-signaling activity. During the development of embryonic ventral epidermis, rho is required for specifying the several cell widths of most ventral ectoderm cells (Mayer and Nüsslein-Volhard, 1988; Raz and Shilo, 1993). However, rho is expressed in only two cell widths of mesectodermal cells at the ventral midline (Bier et al., 1990; Schweitzer et al., 1995a; Sturtevant et al., 1996; Golembo et al., 1996a). The inductive signal mediated by rho for specifying ventral cell fate can act up to several cell diameters from rho-expressing cells. This signal is probably mediated by the sSpi protein in this hypothesis (Schweitzer et al., 1995a; Golembo et al., 1996a,b). The precise molecular mechanism of how Rho protein activates the DER-signaling pathway is still an open question. However, in the case of COP formation, cells that require DER-signaling activity do not seem to be rho-expressing COPs. In rho null mutants, five ato-expressing COPs (data not shown) and five Ch organs remain intact. Therefore, the latter hypothesis proposed by Golembo et al. (1996a) agrees with our present results on COP formation. It is possible that sSpi protein processed from mSpi by the Rho

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**Fig. 4.** Ch organs in mutants with loss of the DER-signaling function. Anti-Cpo immunostaining of LCh5 (filled arrowheads) and V'Ch1 (open arrowheads) of stage 16 embryos. Anterior is to the left and dorsal is to the top. (A) Wild type, (B) flb<sup>2L6S</sup>/flb<sup>2L6S</sup>, (C) Ras<sup>1c2F</sup>/Ras<sup>1c2F</sup>, (D) pm<sup>7848Δ78</sup>/pm<sup>7848Δ78</sup>, (E) yan<sup>yl</sup>/yan<sup>yl</sup>, (F) rho<sup>del1</sup>/rho<sup>del1</sup>. The loss of the DER-signaling pathway, DER (B), Ras<sup>1c</sup> (C) and pm<sup>P2</sup> (D) result in the absence of two Ch organs in LCh5, which is the same phenotype as in the rho loss-of-function phenotype (F). The loss of the intracellular negative regulator (yan) function causes the extra Ch organ in LCh5 (E).

**Fig. 5.** (A-C) argos expression in rho and pm<sup>P2</sup> mutants in stage 11 embryos. Anterior is to the left and dorsal is to the top. (A) Wild type, (B) rho<sup>del1</sup>/rho<sup>del1</sup>, (C) pm<sup>7848Δ78</sup>/pm<sup>7848Δ78</sup>. The argos gene is expressed in cells surrounding COPs. All arrowheads indicate the same COP as judged by its relative position to the tracheal pit (indicated by T). Note that argos transcripts are also expressed in undifferentiated ectoderm cells (arrows in A) above a rho-expressing COP (out of the focal plane). This argos expression is completely absent in the rho null mutant (B) and is greatly reduced in the pm<sup>P2</sup> null mutant (C). (D) A late stage 11 embryo double-stained with argos antisense RNA probe (blue) and anti-Ase antibody (brown). The open arrow indicates a rho-expressing COP located in the middle (indicated by 3 in Fig. 2C). An argos-expressing cell (closed arrow) next to the rho-expressing COP starts to express Ase in its nucleus. Another argos-expressing cell (arrowhead) does not express Ase.
protein diffuses from five rho-expressing COPs to the neighboring cells including undifferentiated ectodermal cells.

Argos functions as a lateral inhibitor in the development of three argos-expressing COPs

rho expression in ato-expressing COPs is likely to induce different levels of DER-signaling activity in neighboring cells. The threshold of DER-signaling activity is one of the most important factors for the differentiation of three argos-expressing COPs. An overdose of either the inductive signal (overexpression of rho) or the inhibitory signal (overexpression of argos; Okabe et al., 1996) caused changes in the number of Ch organs. Thus, not only the intercellular factor but also a balance between inductive and inhibitory signals are necessary to regulate the precise number of COPs. argos expression that depends on DER-signaling activity may be required to generate a locally steep gradient of DER-signaling activity by constructing a negative feedback loop (Golembo et al., 1996b).

We also showed that the three DER-dependent COPs originated from argos-expressing cells. As the rho-expressing COP is next to an argos and Ase double-positive cell and an argos single-positive cell, Argos may have prevented the continuance of DER-signaling activation in the argos single-positive cell from adapting a neural fate. Based on their positions, these argos-expressing cells had once expressed ato in a proneural cluster (Fig. 6A). However, lateral inhibition mediated by neurogenic genes had prevented these ato-expressing undifferentiated ectodermal cells that are to express argos later from becoming COP (Goriely et al., 1991). Future studies may elucidate the correlation between this lateral inhibition mediated by neurogenic genes and the inductive signal mediated by Rho.

Fig. 6. (A) Lineage analysis using the yeast flp/FRT method. A brief heat shock treatment randomly causes a flpase-mediated recombination between two flpase recombination target (FRT) sites. After a recombination, the actin promoter is fused to the lacZ gene directly and the intervening sequence containing the termination codon is eliminated. Observation of lacZ clones of Ch organs permits the determination of the cell lineage of individual COPs. (B) Lateral view of a stage 16 embryo double-stained with mAb22C10 and anti-β-galactosidase antibody after the flp/FRT method. A Ch organ clone including an attachment cell (a) in LCh5 is shown. Note that no other epidermal clone was observed in the same abdominal hemisegment. c, cap cell; s, scolopale cell; n, neuron; l, ligament cell. (C) Summary of results of the COP lineage analysis. We observed 40 Ch organs composed of four lacZ-labeled cells. The numbers of clones that were obtained in this experiment are indicated at the bottom. We never observed more than two labeled Ch organs simultaneously in one hemisegment.
genic genes is also involved in R8 selection, it corresponds to (Jarman et al., 1994). As lateral inhibition mediated by neurogenic genes is a proneural gene as a lateral inhibitor for preventing neighboring cells from being recruited as extra COPs.

In conclusion, the final eight COPs in each abdominal segment are derived in two phases as follows (Fig. 7). The first phase is the selection of five ato-expressing COPs from ato-expressing proneural clusters, which is mediated by the action of the ato gene as a proneural gene and lateral inhibition through neurogenic genes. The first phase does not require DER-signaling activity. The second phase is the induction of three argos-expressing COPs, which require DER-signaling activity. The transition point from the first to the second phase appears to be triggered by the onset of rho expression in the first five ato-expressing COPs. rho gene products in ato-expressing COPs provide inductive signals that can induce the differentiation of neighboring cells into argos-expressing COPs by activation of their DER-signaling pathway. These DER-activated cells secrete Argos protein subsequently, and prevent neighboring cells from becoming extra COPs by providing a feedback loop as a second lateral inhibition.

What is the difference between ato and AS-C function?
In contrast to the determination of Es organ precursors, the number of COPs seems to be regulated by a relatively more complicated machinery. The question is whether the difference in their respective regulatory machinery is due to a difference in the proneural genes involved, since the neurogenic genes are common to formations of both types of SOPs. ato is a proneural gene required not only for Ch organs but also for adult photoreceptor cells (Jarman et al., 1994, 1995). The development of photoreceptor cells seems to share some common regulatory mechanisms with that of COPs. ato is expressed in the anterior edge of the morphogenetic furrow that appears to consist of proneural clusters for photoreceptor cells, and later ato is refined to photoreceptor R8, which appears as the first of eight photoreceptors within each pre-ommatidial cluster (Ready, 1989; Jarman et al., 1994, 1995). Although the loss of ato function causes a lack of all photoreceptor cells, ato mosaic analyses showed that ato is specifically required for the selection of photoreceptor cell R8, which then induces the sequential formation of the other photoreceptors R1-R7 (Jarman et al., 1994). As lateral inhibition mediated by neurogenic genes is also involved in R8 selection, it corresponds to the first phase of COP development (Cagan and Ready, 1989; Baker et al., 1990, 1996; Baker and Zitron, 1995). The rho gene product in ato-expressing photoreceptor R8 is probably involved in the activation of the DER-signaling pathway in neighboring cells to cause them to differentiate into the outer photoreceptors R1-R6 (Freeman et al., 1992b). In this sequential photoreceptor induction, the argos gene operates to prevent supernumerary photoreceptor development by being a lateral inhibitor of the DER-signaling pathway (Freeman et al., 1992a; Kretzschmar et al., 1992; Okano et al., 1992; Schweitzer et al., 1995b; Sawamoto et al., 1996). This sequential induction of R1-R6 by DER-signaling activity corresponds to the second phase of COP development. For the reasons mentioned above, it is possible that ato has at least two different functions in the formation of both photoreceptors and COP. One is a function as a proneural gene like AS-C in Es organ precursor formation. The other function is required for expressing the inductive signal for neural differentiation of neighboring cells.

The question then arises of whether the three argos-expressing COPs require the activity of the proneural gene for their differentiation, because they do not express the proneural genes ato or AS-C. One possibility is the presence of unidentified proneural gene(s) required for their formation. Alternatively, it is possible that a transient ato expression in their precursor cells (cells in proneural clusters) and the late local inductive signal that activates DER-signaling is sufficient to induce pan-neuronal gene expressions such as ase. Further investigation will provide clarification.

The authors express their thanks to Drs Kazunobu Sawamoto, Shingo Yoshikawa and Takeshi Asakawa for enlightening discussions, to Dr Yasushi Hiromi for technical instructions, to Drs Ethan Bier, Yuh Nung Jan, Gerald Rubin, Hugo Bellen and Kenichi Kimura for mutant flies, cDNA and antibodies, Drs. Miyuki Yamamoto, Tadashi Uemura and Shigoe Hayashi for their valuable comments on the manuscript and to the entire staff of the Okano Laboratory for their encouragement. We are also grateful to the Bloomington Stock Center for providing the strains used in this study. This work was supported by grants to H.O. from the Ministry of Education, Science, and Culture of Japan and TARA Okano project, and by CREST (Core Research for Evolutional Science and Technology) of Japan Science and Technology Cooperation (JST).

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(Accepted 29 November 1996)