Association of tracheal placodes with leg primordia in *Drosophila* and implications for the origin of insect tracheal systems

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Adaptation to diverse habitats has prompted the development of distinct organs in different animals to better exploit their living conditions. This is the case for the respiratory organs of arthropods, ranging from tracheae in terrestrial insects to gills in aquatic crustaceans. Although *Drosophila* tracheal development has been studied extensively, the origin of the tracheal system has been a long-standing mystery. Here, we show that tracheal placodes and leg primordia arise from a common pool of cells in *Drosophila*, with differences in their fate controlled by the activation state of the *wingless* signalling pathway. We have also been able to elucidate early events that trigger leg specification and to show that cryptic appendage primordia are associated with the tracheal placodes even in abdominal segments. The association between tracheal and appendage primordia in *Drosophila* is reminiscent of the association between gills and appendages in crustaceans. This similarity is strengthened by the finding that homologues of tracheal inducer genes are specifically expressed in the gills of crustaceans. We conclude that crustacean gills and insect tracheae share a number of features that raise the possibility of an evolutionary relationship between these structures. We propose an evolutionary scenario that accommodates the available data.

KEY WORDS: Tracheae, Appendage primordia, Gills, *wingless* signalling, Distal-less (Dll), buttonhead (btd), trachealless (trh), ventral veinless (vvl)

INTRODUCTION

A crucial step in the colonization of terrestrial habitats has been the evolution of specialized respiratory organs that allow efficient gas exchange and minimize water loss. The main respiratory organs in insects are the tracheae, comprising a network of tubes that bring air deep into the body (Snodgrass, 1935; Mill, 1985). In *Drosophila*, as in other insects, the tracheal system is a complex tubular network that arises from the tracheal placodes, clusters of ectodermal cells that appear on either side of thoracic and abdominal embryonic segments. Tracheal cells are specified by the activity of a set of *‘tracheal inducer genes’* that includes the transcription factors *trachealless (trh)* and *ventral veinless (vvl)*, whose expression is controlled by genes that specify positional cues along the embryonic body axes (Isaac and Andrew, 1996; Wilk et al., 1996; Boube et al., 2000). The cells of each cluster invaginate and migrate in a stereotypic pattern to form each of the primary tracheal branches (Manning and Krasnow, 1993).

In recent years, many of the genes that are required for the specification of the tracheal cells have been identified (Ghahrial et al., 2003), however, not much attention has been given to the evolutionary origin of these cells. It is believed that in the common ancestors of all arthropods, specialised parts of appendages had a major role in respiration and osmoregulation, acting as gills (Brusca and Brusca, 1990; Budd, 1996). Indeed, this close association between respiratory organs and appendages is maintained currently in many crustaceans, which are the closest living relatives of insects (Regier and Shultz, 1997; Boore et al., 1998; Mallatt et al., 2004).

To investigate the origin of tracheal cells, we have asked whether these may also arise in association with the cells that give rise to appendages in a present day insect like *Drosophila*. We have found that indeed the tracheal placodes and leg primordia arise from a common pool of cells in *Drosophila*, and that the decision between these two fates is controlled by the activity of the *wingless* signalling pathway. By manipulating the genetic program that controls leg specification, we have been able to show that, even in the abdomen, tracheal primordia develop in close association with cryptic appendage primordia. These results point to a close relationship between the tracheal and leg fates, and suggest some interesting similarities with the appendage-associated gills of aquatic crustaceans. To investigate these similarities further, we have cloned homologues of the tracheal inducer genes and studied their expression patterns in two divergent groups of crustaceans. We argue that crustacean gills and insect tracheae, hitherto considered to be independent systems for gas exchange, may share a number of features in their developmental origin and specification.

MATERIALS AND METHODS

Fly strains

We have used *w{CG4}X4* as a *w* null allele. The 1-eve-1 line (Perrimon et al., 1991) is a lacZ insertion in the *trh* gene. Expression of *btd* and *Dll* was induced with the UAS/GAL4 system (Brand and Perrimon, 1993), using a UAS-*btd* line (Schock et al., 1999) and a UAS-Dll line (Forfinkiel and Guerrero, 1997), and a *btd*-GAL4 line as a driver (Estella et al., 2003). To induce ectopic expression of *wg*, we used a *nullo-Gal4* (from W. Gehring, Basel, Switzerland) and a UAS-wg line (Lawrence et al., 1995). We used *ptc-GAL4* (Sperchier et al., 1994) to drive the expression of UAS-TCF{\textsuperscript{DN}} (van de Wetering et al., 1997) and UAS-Arm\* (Pai et al., 1997) in *Drosophila* embryos.

*Drosophila* immunostaining and in situ hybridisation

We used the following primary antibodies: a mAb2A12 monoclonal antibody (1:5-1:10, from the Developmental Studies Hybridoma Bank, University of Iowa), which recognises an epitope from the lumen of the...
tracheal tree, and an antibody specific for β-gal (Cappel, 1:2000). Embryos were stained according to standard protocols using the Vectastain Elite ABC kit. For immunofluorescence, we used secondary antibodies Alexa488-conjugated goat anti-rabbit (1:200) and Alexa594-conjugated goat anti-mouse (1:200), both from Molecular Probes. Whole-mount in situ hybridisation was carried out with trh and btd anti-sense RNA probes, following the method of Tautz and Pfeifle (Tautz and Pfeifle, 1989), with minor modifications. For immunofluorescence, we used trh anti-sense RNA probes following the procedure described by Wilkie and Davis (Wilkie and Davis, 1998). For antibody labelling followed by in situ hybridisation, we followed the procedure described by Manoukian and Krause (Manoukian and Krause, 1992). Photographs were taken using Nomarski optics or a SP1 Leica confocal microscope.

Preparation of embryonic cuticle
For the analysis of embryonic cuticle, late embryos were removed from the chorion and vitelline membrane, and mounted in a mixture of Hoyer’s medium (van der Meer, 1977) and lactic acid (1:1).

**Artemia, Parhyale and crayfish immunostaining and in situ hybridisation**
We initially cloned a fragment of vvl from Artemia, by PCR from cDNA generated from larval RNA with degenerated oligonucleotides designed from the *Drosophila vvl* sequence. An antibody against the Vvl protein of *Artemia* was generated by injecting rabbits with a His-tagged fragment of the Vvl protein [amino acids 241 to 386 of the previously published sequence (Chavez et al., 1999)]. The serum was then affinity purified on a nickel column. We initially cloned a fragment of vvl and trh from *Parhyale hawaiensis* by PCR from a cDNA library kindly provided by Nipam Patel (University of California, Berkeley), with degenerated oligonucleotides designed from the *Drosophila vvl* and trh sequences. We obtained a 240 bp fragment of the *Parhyale vvl* gene that we used to clone the full-length cDNA from the library. We also obtained a *Parhyale trh* fragment of around 700 bp that encompasses the region of the HLH, the PAS-1 and the PAS-2 domains (corresponding to amino acids 100 to 550 in *Drosophila*). In situ hybridization in *Parhyale* was carried out using a protocol provided by Nipam Patel; the protocol is available upon request. For immunostaining in crayfish embryos, we used the 4D9 monoclonal antibody for Engrailed (Patel et al., 1989) and a polyclonal antibody for Nub/Pdm (Averof and Cohen, 1997). Immunohistochemical staining was carried out as described by Patel (Patel, 1994).

**RESULTS AND DISCUSSION**
**Tracheal placodes arise in close proximity to the leg primordia in *Drosophila***

The *Drosophila* tracheal system has a clearly metameric origin, arising from clusters of cells, on either side of each thoracic and abdominal segment, that express the tracheal inducer genes tracheless (trh) and ventral veinless (vvl) (de Celis et al., 1993; Isaac and Andrew, 1996; Wilk et al., 1996) (Fig. 1B). Conversely, the leg precursors can be recognized as clusters of cells that express the Distal-less (Dll) gene, on either side of each thoracic segment; these will give rise both to the Keilin’s Organs (KOs, the rudimentary legs of the larvae) and to the three pairs of imaginal discs that will give rise to the legs of the adult fly (Cohen, 1993).

To investigate whether there is a direct physical association between the leg and tracheal primordia, we examined *Drosophila* embryos co-stained for the expression of trh and early markers of leg primordia. Although Dll is one of the most commonly used markers for the leg primordia, it is not the earliest gene required for their specification. Instead, a couple of related and apparently redundant genes, buttonhead (btd) and Sp1, act upstream of Dll in the specification of these primordia (Estella et al., 2003). Examining the specification of tracheal cells with respect to btd expression, we observe that tracheal cells appear in close apposition to btd-expressing cells, from the earliest stages of their appearance (by stage 9/early stage 10, Fig. 1E.F). Interestingly, unlike Dll, btd is initially expressed both in the thoracic and abdominal segments, and its expression is restricted to the thoracic segments later, under the influence of the BX-C genes (Estella et al., 2003). Thus, the cells of the respiratory system in *Drosophila* always arise in close proximity to the cells that are fated to give rise to the legs.

**Induction of Keilin’s organs in the abdomen: tracheal primordia are associated with cryptic appendage primordia in the abdominal segments**
To fully endorse this conclusion it is necessary to show that the btd-expressing cells in the abdomen correspond to cryptic leg primordia. This may be a key point because, although many of the genes required for leg development are already known, it has not yet been possible to induce leg development in abdominal segments (except by transforming these segments into thoracic ones). In particular, although the Dll promoter contains BX-C binding sites that repress its expression in the abdominal segments (Vachon et al., 1992), no ectopic appendage has been reported by misexpressing Dll in the abdomen. These observations have lead to some doubts as to whether a leg developmental program is at all compatible with abdominal segmental identity.

As the initial expression of btd in the abdominal segments is downregulated by the BX-C genes, we reasoned that sustained expression of btd might overcome the repressive effect of the BX-C genes and force the induction of leg structures in the abdomen. To test this, we used a btd-GAL4 driver to drive btd expression, expecting that the perdurance of the GAL4/UAS system would ensure a more persistent expression of btd in its endogenous expression domain. We never obtained any sign of ectopic Dll expression or KOs in the abdominal segments, but we observed...
that the increased expression of btd had an effect on the KOs of the thoracic segments, which had more sensory hairs than the three normally found in wild-type KOs (Fig. 2F). Thus, on its own, btd seems unable to overcome BX-C repression of leg development.

One possibility would be that the BX-C genes could suppress appendage development in the abdomen by independently repressing both btd and Dll in this region. To assess this possibility, we used the same btd-GAL4 driver to simultaneously induce the expression of both btd and Dll. Under these circumstances, we observe that KOs develop in otherwise normal abdominal segments (Fig. 2B,D,G); as in the previous experiment, the newly formed KOs have more than three sensory hairs. These results suggest that expression of btd and Dll in the btd-expressing abdominal primordia is sufficient to induce the development of leg structures in the abdomen, overcoming the repressive effect of the BX-C genes. Furthermore, these results demonstrate that these clusters of btd-expressing cells in the abdomen are indeed cryptic leg primordia. These results clearly show that tracheal cells are specified in close proximity to the leg primordia, in both thoracic and abdominal segments.

A leg-tracheal equivalence group: wingless signalling provides a genetic switch for the specification of leg versus tracheal fate

Previous results have shown that the leg primordia are specified straddling the segmental stripes of wingless (wg) expression in the early embryonic ectoderm (Cohen et al., 1993), whereas tracheal cells are specified in between these stripes (de Celis et al., 1995). To investigate whether wg might play a role in determining the fate of these primordia, we studied what happens when the normal pattern of wg expression is disrupted. We find that, in wg mutant embryos, trh and vvl from the earliest stages of their expression are no longer restricted to separate clusters of cells; instead larger patches of expression add up to a continuous band of cells running along the anteroposterior axis of the embryo (Fig. 3C) (de Celis et al., 1995), while btd expression is suppressed in this part of the embryonic ectoderm (Fig. 3D) (Estella et al., 2003). Conversely, ubiquitous expression of wg suppresses trh expression (Fig. 3E), while causing an expansion of btd expression along the embryo (Fig. 3FL). Restricted activation or inactivation of the wg pathway by the expression of a constitutive form of armadillo or a dominant-negative form of dTCF, respectively, are also able to specifically induce or repress trh and btd expression (Fig. 3G-J). trh/vvl and btd seem to respond independently to wg signalling and there is no sign of cross-regulation among them, as btd expression is normal in trh vvl double mutants, and trh and vvl expression is normal in mutants for a deficiency uncovering btd and Sp1 (data not shown).

The role of wg as a repressor of the tracheal fate is further illustrated by looking at the behaviour of transformed cells: the clusters of cells that have lost btd expression and gained trh and vvl expression in wg mutant embryos begin a process of invagination that is characteristic of tracheal cells (Fig. 3K). Furthermore, these cells also express the of(stumps – FlyBase) gene, a target gene of both trh and vvl in the tracheal cells (Boube et al., 2000) (data not shown). Although further development of these cells is hard to ascertain because of gross abnormalities in wg embryos, these results indicate that they have been specified as tracheal cells. Thus, wg appears to act as a genetic switch that decides between two mutually exclusive fates in this part of the embryonic ectoderm: the tracheal fate, which is followed in the absence of wg signalling; and the leg fate, which is followed upon activation of the wg pathway (Fig. 3M). Given that there are no cell lineage restrictions setting apart the cells of the tracheal and leg primordia (Meise and Janning, 1993), these two cell populations could be considered as a single equivalence group, with the differences in their fate controlled by the activation state of the wg signalling pathway.

Crustacean homologues of tracheal inducer genes are expressed in appendage-associated gills

A link between respiratory organs and appendages is also found in many primitively aquatic arthropods, like crustaceans, where gills typically develop as distinct dorsal branches (or lobes) of appendages called epipods (Brusca and Brusca, 1990). Following our observations, which suggest a link between respiratory organs and appendages in Drosophila, we decided to examine whether
Further similarities could be found between insect tracheal cells and crustacean gills. Specifically, we considered whether homologues of the tracheal inducing genes might have a role in the development of appendage-associated gills in crustaceans.

We used RT-PCR to clone fragments of the vvl and trh homologues from Artemia franciscana and from Parhyale hawaiiensis, representing two major divergent groups of crustaceans (members of the branchiopod and malacostracan crustaceans, respectively). In the case of Artemia vvl, we cloned a fragment that corresponds to the APH-1 gene previously reported by Chavez et al. (Chavez et al., 1999) and generated an antibody for immunohistochemical staining in developing Artemia larvae. We observe that Artemia Vvl is initially absent from early limb buds; it becomes weakly and uniformly expressed while the limb is developing its characteristic branching morphology, and becomes strongly upregulated in one of the epipods as its cells begin to differentiate (Fig. 4A,B). Uniform weak expression persists in mature limbs, but expression levels in the epipod are always significantly higher. The trh homologue from Artemia has previously been studied by Mitchell and Crews (Mitchell and Crews, 2002), and its expression appears to be restricted to the same epipod as Vvl. Similarly, we have cloned homologues of vvl and trh from Parhyale hawaiiensis and have studied their expression by in situ hybridization. Both genes are specifically expressed in the epipods of developing thoracic appendages (Fig. 4C-E). Besides epipods, the Artemia trh and vvl homologues are also expressed in the larval salt gland, an organ with osmoregulatory functions during early larval stages of Artemia development (Chavez et al., 1999; Mitchell and Crews, 2002).

**Implications for the origin of insect tracheal systems**

What is the significance of the two Drosophila tracheal inducer genes being specifically expressed in crustacean epipods/gills? One possibility is that the expression of these two genes was acquired independently in insect tracheae and in crustacean gills. Alternatively, tracheal systems and gills may have inherited these expression patterns from a common evolutionary precursor, perhaps a respiratory/osmoregulatory structure that was already present in the common ancestors of crustaceans and insects.

The latter possibility is considered unlikely by conventional views, because of the structural differences between gills and tracheae (external versus internal organs, discrete segmental organs versus fused network of tubes), and the difficulty to conceive a smooth transition between these structures. Yet, analogous transformations have occurred during arthropod evolution: tracheae can be organized as large interconnected networks or as isolated entities in each segment (as in some apterygote insects), invagination of external respiratory structures is well documented among groups that have made the transition from aquatic to terrestrial environments (terrestrial crustaceans, spiders and scorpions), and conversely evagination of respiratory surfaces is common in animals that have returned to an aquatic environment (tracheal gills or blood gills in aquatic insect larvae) (Snodgrass, 1935; Mill, 1985; Brusca and Brusca, 1990). A very similar (but independent) evolutionary transition is, in fact, thought to have occurred in arachnids, where gills have been internalised to give rise to book lungs, and these in turn have been modified to give rise to tracheae in some groups of spiders (Lankester, 1885; Purcell, 1910; Damen et al., 2002). Thus, a relationship between insect tracheae and crustacean gills is plausible.

A particular type of epipod/gill has also been proposed as the origin of insect wings (Wigglesworth, 1976; Kukalova-Peck, 1983), a hypothesis that has received support from the specific expression...
of the pdm/nubbin (nub) and aporterous (ap) genes – that have wing-specific functions in Drosophila – in a crustacean epipod (Averof and Cohen, 1997). In fact, the Artemia nub and ap homologues are expressed in the same epipod as trh and vvl, raising questions as to the specific relationship of this epipod with either tracheae or wings. A resolution to this conundrum becomes apparent when one considers the different types of epipods/gills found in aquatic arthropods, and their relative positions with respect to other parts of the appendage.

The primary branches of arthropod appendages, the endopod/leg and exopod, develop straddling the anteroposterior (AP) compartment boundary, which corresponds to a widely conserved patterning landmark in all arthropods (Martinez-Arias and Lawrence, 1985; Patel et al., 1989a; Basler and Struhl, 1994; Damen, 2002). Different types of epipods/gills, however, differ in their position with respect to this boundary. For example, in the thoracic appendages of the crayfish, some epipods develop spanning the AP boundary [visualized by engrafted (en) expression running across the epipod], whereas others develop exclusively from anterior cells (with no en expression; Fig. 4F). Given that wing primordia comprise cells from both the anterior and posterior compartments, wings probably derived from structures that were straddling the AP boundary. Conversely, given that tracheal primordia arise exclusively from cells of the anterior compartment (anterior to en and even wg-expressing cells) (de Celis et al., 1995), it seems probable that tracheal cells evolved from a population of cells that was located in the anterior compartment. In this respect, it is interesting to note that the former type of epipods express nub, whereas the latter do not (Fig. 4G).

In summary, we would like to suggest that the ancestors of arthropods had specific areas on the surface of their body that were specialized for osmoregulation and gas exchange. Homologues of trh and vvl were probably expressed in all of these cells and played a role in their specification, differentiation or function. Some of these structures were probably associated with appendages, in the form of epipods/gills or other types of respiratory surfaces. A particular type of gill, straddling the AP compartment boundary, is likely to have given rise to wings (Averof and Cohen, 1997), whereas respiratory surfaces arising from anterior cells only may have given rise to the tracheal system of insects. Confirmation of this hypothetical scenario may ultimately come from the discovery of new fossils, capturing intermediate states in the transition of insects from an aquatic to a terrestrial lifestyle.

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