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Spitz/EGFr signalling via the Ras/MAPK pathway mediates the induction of bract cells in *Drosophila* legs

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SUMMARY

In the development of *Drosophila*, the activation of the EGFr pathway elicits different cellular responses at different times and in different tissues. A variety of approaches have been used to identify the mechanisms that confer this response specificity. We have analysed the specification of bract cells in *Drosophila* legs. We observed that mechanosensory bristles induced bract fate in neighbouring epidermal cells, and that the RAS/MAPK pathway mediated this induction. We have identified Spitz and EGFr as the ligand and the receptor of this signalling, and by ubiquitous expression of constitutively activated forms of components of the pathway we have found that the acquisition of bract fate is temporally and spatially restricted. We have also studied the role of the *poxn* gene in the inhibition of bract induction in chemosensory bristles.

Key words: *Drosophila*, Leg, Bract, *EGFr*, *spitz*, *poxn*

INTRODUCTION

In the development of multicellular organisms, intercellular signalling plays a major role in both the control of cell proliferation and in the determination of different cell fates. So far only a small number of signal translation pathways have been confirmed to be involved in these processes. The role of epidermal growth factor receptor (EGFr) signalling through the RAS/MAPK cascade is particularly relevant (reviewed by Moghal and Sternberg, 1999). In *Drosophila*, the EGFr is required for the control of cell proliferation and/or cell viability in imaginal disc cells. EGFr signalling also plays a role in the specification of many cell types: in the eye it is the main trigger for the differentiation of all cell types, and in the thorax its function is required in the specification of wing veins and notum bristles. The EGFr is also required in oogenesis and in early embryo development (reviewed by Schweitzer and Shilo, 1997).

One of the main objectives of the analysis of cell signalling is to understand how the reiterative activation of a receptor in different tissues and at different times can elicit different cellular responses. The experimental evidence does not suggest a simple answer, and rather indicates a high complexity in the mechanisms involved. In *C. elegans*, the LET-23 tyrosine kinase receptor is required in several tissues and it has recently been revealed to signal through the RAS/MAPK in some tissues, but via inositol triphosphate in the germline (Cladinin et al., 1998). In PC12 cells, stimulation by EGF results in a transient activation of MAPK, but stimulation by NGF results in prolonged activation and triggers different responses (Marshall, 1995). In *Drosophila*, different thresholds of MAPK activation in the embryo (Greenwood and Struhl, 1997) and in the developing eye (Halfar et al., 2001) also trigger different cellular responses. The integration of signals from several pathways provides another mechanism for conferring specificity. The Delta/Notch and RAS/MAPK pathways act in combination to specify R7 fate in the *Drosophila* eye (Tomlinson and Struhl, 2001), but antagonistically to specify bristles in the notum (Culí et al., 2001). EGFr signalling also acts antagonistically to Wg signalling in larval epidermis (O'Keefe et al., 1997), and to Dpp signalling to establish distinct cell fates in tracheal placodes (Wappner et al., 1997). Finally, the expression of tissue-restricted transcription factors provides developmental contexts that confer specific responses. This is the case for *LIN-31* in *C. elegans*, which is specifically involved in vulva development (Tan et al., 1998), and for the *Drosophila* gene *yan*, an inhibitory ETS transcription factor required in the eye disc but not in other imaginal discs (Lai and Rubin, 1992).

In this report, we describe a new model of cell fate determination in development involving the RAS/MAPK pathway: the specification of bract cells in the *Drosophila* epidermis. The cuticle of the adult fly is covered by an array of different kinds of sensory organs. Most of these are large or small bristles that are arranged in very precise patterns. They can also be classified according to their sensitivity to external stimuli as mechanosensory (MB) or chemosensory (ChB) bristles. In the legs and in the proximal costa of the wing, MB occur in association with a cell called bract (Hannah-Alava, 1958). Bracts are clearly distinguishable from the trichomes

that the rest of the epidermal cells differentiate in adult cuticle. Detailed analysis of the fine structure of the bract reveals that it is a single epidermal cell (Reed et al., 1975; Walt and Tobler, 1978). Early experiments, in which imaginal disc cells are dissociated and re-aggregated, showed that after aggregation bract cells always appear associated to certain bristles. This suggests that the acquisition of bract fate requires the presence of a bristle nearby, and an inductive action carried out by the bristle over a neighbouring epidermal cell has been proposed (García-Bellido, 1966; Tobler, 1966).

We have analysed the mechanism of the specification of bract fate and the involvement of neighbouring bristles. Our results indicate that the inductive signal that activates bract fate is mediated by the RAS/MAPK signalling pathway. We propose a model by which, in early pupal development, the expression of the *rhomboid* gene in sensory organ precursors (SOPs) activates the TGF-α homologue encoded by the gene *spitz*, which then signals through the EGFr to activate the RAS/MAPK pathway. The activation of the pathway in a neighbouring epidermal cell specifies bract fate. The number of bracts per bristle is controlled by a lateral inhibition mechanism mediated by the *argos* gene. By expressing constitutively activated forms of the RAS/MAPK pathway, we have found that the competence to acquire bract fate is temporally and spatially restricted, supporting the role of the developmental context in determining response specificity. The absence of bracts in chemosensory bristles is explained by the inhibitory action of the *poxn* gene on the processing of Spitz protein.

MATERIALS AND METHODS

Drosophila strains

The following fly strains were used in this work: spi^{IIA14} (Rutledge et al., 1992), $rho1^{de11}$ (Bier et al., 1990), $aos^{\Delta 15}$ (Kretzschmar et al., 1992), top^{4A} (Price et al., 1989), $ras1^{e2F}$ (Simon et al., 1991), raf^{EA75} (Melnick et al., 1993), $gap1^{B2}$ (Gaul et al., 1992), Sos^{X122} (Rogge et al., 1991), rl^{1} and $Df(2R)rl^{10a}$ (Hiliker, 1976), *sple* and $In(1)sc^{10-1}$ (Lindsley and Zimm, 1992), *hsp70-poxn* (Nottebohm et al., 1992), *hsp70*-GAL4 (Brand et al., 1994), dpp^{disk}-GAL4 (Staehling-Hapton et al., 1994), *neu*-GAL4 (Jhaveri et al., 2000), *sca*-GAL4, UAS-*sSpi* (Schweitzer et al., 1995), UAS-*rho1* and UAS-*S* (Golembo et al., 1996), UAS-*aos* (Freeman, 1994), UAS-activated *Raf* (Martín-Blanco et al., 1999), UAS-*Nintra* (Struhl and Adachi, 1998).

Clonal analysis

Fly strains and strategies for clonal analysis of the RAS/MAPK pathway are described in (Díaz-Benjumea and Hafen, 1994). The number of clones scored in different clonal analyses is 15 spi^{IIA14}, 8 top^{4A}, 10 $ras1^{e2F}$, 9 raf^{EA75}, 16 Sos^{X122} and 5 $rho1^{de11}$. For cell lineage analysis, *y FLP122* P{Act5C>CD2>GAL4} / FM7; UAS-y^{+} females were crossed with wild-type males, and F_1 larvae at 72±6 hours after puparium formation (APF) were heat shocked for 7 minutes at 36°C. Adult males from the progeny were mounted in araldite and their legs scored for the presence of y^{+} clones. Several clones per leg were found in all the scored flies.

Heat shock experiments

For overexpression experiments, larvae were grown at 18°C, and when they started to pupate, pupae were collected and classified into the following stages: 0-4 hours, 4-8 hours, 8-12 hours and >12 hours APF (García-Bellido, 1971). Pupae were then transferred to new tubes and heat shocked for 30 minutes at 37°C in a water bath. After heat shock they were kept at 18°C, and adults with the appropriate genotype were mounted for cuticle analysis under the compound microscope. No major effects were found in wild-type flies heat shocked under the same experimental conditions as a control (Held, 1990).

Preparation of adult cuticle

Flies were dissected in glycerol/ethanol, fat was removed in 10% KOH, and the cuticle was dehydrated in ethanol, transferred to acetone and mounted in araldite.

RESULTS

Bracts are single cell epidermal structures

Drosophila legs are covered by a constant and leg-specific pattern of different types of external sensory organs, mainly MB and ChB. Bristles on the legs can be classified by the presence of bracts. Bracts are small epidermal structures that appear associated to MB in specific places on adult femur, tibia and the tarsal segments of all legs. Bracts appear on the proximal side of the bristles, and share the same polarity (Fig. 1A,B). Bracts are also present in the proximal costa of the wing, showing the same morphology as in the leg (Fig. 1C,D).

Are bristle and bract related by lineage? Sensory organ precursors (SOPs) undergo a specific pattern of cell divisions that give rise to four cells: two epidermal cells, the shaft and socket, and two neural cells, a neurone and a sheath cell (Bate, 1978; Jan and Jan, 1993). Previous clonal analyses of leg disc suggested a lack of lineage relationship between bristles and bracts (Tokunaga, 1962; Lawrence et al., 1979). We have confirmed these results by labelling clones of cells induced in early third instar larvae. These clones were induced with a flip-out cassette that produces a high frequency of clones (see Materials and Methods, for details). Fig. 1E,F shows clones marked with the y^{+} marker in a y^{-} background. Clones can include either the bristle or the bract. As these clones have been induced 48 hours before bristle specification, this result indicates that the bract cell does not belong to the SOP lineage.

The RAS/MAPK pathway mediates the induction of bract fate

It has been proposed that the determination of bracts might be related to an inductive action by its corresponding bristle. We analysed the role of the RAS/MAPK pathway as a mediator of this cell signalling. We studied the pattern of bracts in flies carrying viable mutant alleles of several genes of the RAS/MAPK pathway. Viable combinations of loss-of-function alleles of the *rolled* gene (MAPK) show a complete lack of bracts in wings and legs (Fig. 2A). The *gap1* gene encodes a putative Ras GTPase activating protein that acts as a negative regulator of the MAPK pathway (Gaul et al., 1992). Flies homozygous for the loss-of-function allele $gap1^{B2}$ have extra bracts on legs and wings (Fig. 2B). These extra bracts were always associated with MB and occurred adjacent to the wild-type bract. Both results indicate that the RAS/MAPK pathway mediates the activation of bract fate.

To confirm these results and to identify the ligand and receptor that activate RAS/MAPK signalling, we analysed more severe loss-of-function alleles of several components of

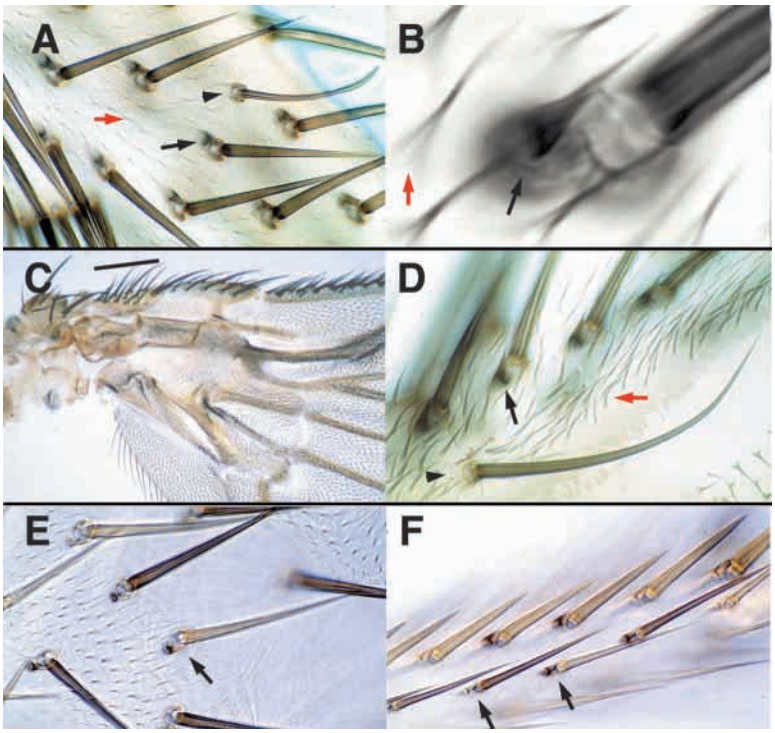

Fig. 1. Bract-cell morphology, distribution and lineage. (A,B) Adult leg showing mechanosensory bristles with the associated bract (black arrows), bract-less chemosensory bristles (arrowhead) and trichomes (red arrows). (C) The wing bracts are restricted to the proximal costa (bar). (D) High magnification of the area labelled in C showing mechanosensory (arrow) and bract-less chemosensory bristles (arrowhead). (E,F) Cell-lineage analysis shows that bracts and bristles are not related by lineage: y^+ clones in a y^- background. Arrows indicate sets of bracts and bristles with different labelling. In all figures, photos that show legs correspond to tibia or basitarsus, apart from Fig. 4A-C and Fig. 5B,F in which, for better resolution, the femur is shown.

the RAS/MAPK pathway. Several receptors with tyrosine kinase activity that act upstream of this pathway have been characterised in *Drosophila* (Perrimon, 1994). The *Drosophila* homologue of the epidermal growth factor receptor, EGFr, is ubiquitously expressed and is required in the development of several imaginal discs. Of the several ligands that signal through this receptor, the *Drosophila* TGFα homologue encoded by the gene *spitz* (*spi*) is the most obvious candidate, as *spi* is also ubiquitously expressed in imaginal discs (Rutledge et al., 1992; Schweitzer et al., 1995). Others genes that have been identified as components of this signalling pathway are *Son of sevenless* (*Sos*), a guanine-nucleotide releasing factor (Rogge et al., 1991), *Ras*, and *Raf*. Owing to their early requirement, strong loss-of-function alleles of these genes are embryonic lethal, so we induced, by mitotic recombination, clones of mutant cells in early larval development. Clones of all mutant alleles, scored in both legs and wings, lacked bracts, but bristles were not affected (Fig. 2C-F). This indicates that the acquisition of bract fate requires activation of the RAS/MAPK pathway through Spi/EGFr and, as bristles are not affected in these clones, that the signalling probably originates in bristle cells.

Spatial competence in the activation of bract fate

Although there are MBs at many places on the adult fly cuticle, bristles with bracts are only found in specific sites on wings and legs. This suggests a spatial restriction that limits the competence to acquire bract fate. This might occur for two reasons. First, away from these areas, bristles might not activate the signal; and second, cells outside these areas might not be competent to adopt the bract fate. To identify the spatial field of competence for activation of bract fate we used the UAS/GAL4 system (Brand and Perrimon, 1993) to express an activated form of the Raf protein ubiquitously (Raf*) (Martín-Blanco et al., 1999). This provides a signal-independent way to activate the RAS/MAPK pathway. Ectopic expression of Raf*, under the control of several GAL4 drivers, produces ambiguous results as the pattern of leg bristles is affected, and bracts are either missing or supernumerary in the same experiments. This may be caused by the fact that the *argos* gene, a EGFr ligand that acts as a competitive inhibitor of other ligands, is itself a target of the pathway (Freeman et al., 1992). To avoid this problem, we induced pulses of UAS-*Raf** expression with a *hsp70*-GAL4 construct (Brand et al., 1994). Early pupae were collected and heat shocked for 30 minutes at 37°C. After this heat shock pupae complete development but the individuals die as pharate adults. In these flies, large territories of trichomes were transformed into bracts. In legs, from

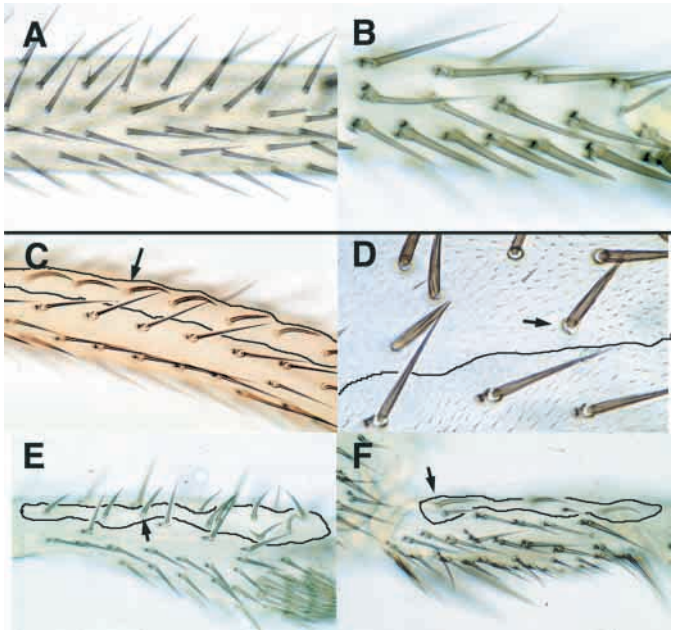

Fig. 2. The RAS/MAPK pathway mediates the induction of bracts. (A) *rolled*^{1}/*rolled*^{10A} leg showing a complete lack of bracts. (B) *gap1*^{B2} leg. Mechanosensory bristles have two or more bracts; chemosensory bristles are not affected. (C-F) Clones of cells mutant for *spi* ^{IIA14}, *EGFr*^{4A}, *Dras*^{e2F} and *Draf*^{EA75}. Clones are outlined and cells are marked with *forked* (C,E,F) or *pawn* (D). All bristles within clones are bract-less (arrows).

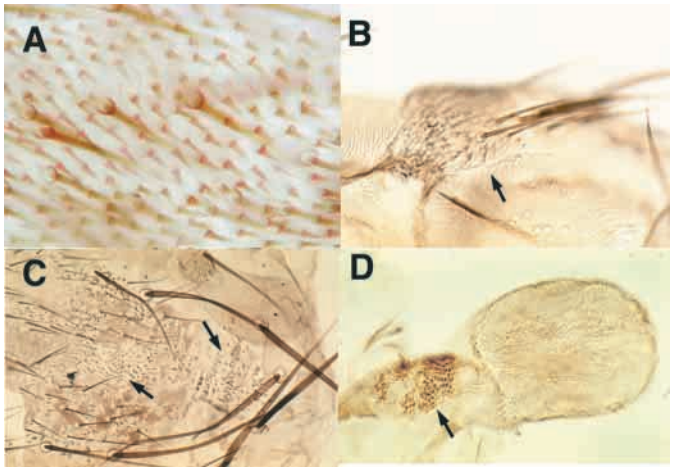

Fig. 3. The competence to specify bracts is spatially restricted. *hsp70*-GAL4/UAS-*Raf* * pupae heat shocked for 30 minutes at 37°C. (A) In the leg, all trichomes are transformed into bracts (compare with Fig. 1A). (B) In the wing, only cells in the proximal costa (arrow) are transformed (compare with Figs. 1C,D). (C) In the notum, many bracts (arrows) appear among dorsocentral bristles. (D) In the haltere, only a region of the pedicellum shows extra bracts. In A,B,D, distal is rightwards. In C, anterior notum is left.

the proximal femur to the claw, all trichomes were transformed into bracts (Fig. 3A). In the notum, the region between the dorsocentral bristles also differentiated multiple bracts (Fig. 3C). In wings, only the cells of the dorsal and ventral proximal costa differentiated as bracts (Fig. 3B); the rest of wing cells were not affected, apart from a small area in the dorsal hinge that also differentiated a patch of bracts. In the halteres, multiple bracts covered the dorsal and ventral anterior pedicelum (Fig. 3D). [It is noteworthy that this area, which normally lacks bristles, develops bristles with bracts in homeotic transformations caused by mutation of the *Ubx* gene (Morata and García-Bellido, 1976).] The prothorax also differentiated a small patch of bracts. In the head, bracts were found at the base of the arista, and in the gena and the supraorbital region. The size and shape of bracts was constant in different parts of the fly. We conclude that in these areas epidermal cells are competent to differentiate as bracts if the RAS/MAPK pathway is activated at the appropriate time.

Temporal restriction in the activation of bract fate

To investigate if a temporal window restricts the acquisition of bract fate, we analysed the phenotype of *hsp70*-GAL4/UAS-*Raf** flies heat-shocked at different stages of pupal development. Pupae were collected, classified from 0-4, 4-8, 8-12 and >12 hours APF, and heat shocked under the same conditions described above. Flies heat shocked at around 8 hours APF differentiated a large number of bracts (Fig. 4C), with almost all the trichomes from the above-defined competent areas transformed. Heat shock at later stages yielded a reduced density of bracts, and earlier heat shock produced a pattern of regions of normal trichomes surrounding patches of bracts (Fig. 4B). Areas covered by bracts were mainly areas among bristles, which suggests that at this time (4-8 hours APF), SOP cells are inhibiting, downstream of Raf, the competence of epidermal cells to become bracts.

The Delta/Notch (D1/N) pathway mediates the process whereby a single cell from the cluster that expresses proneural genes goes on to become an SOP (Simpson, 1990). To test whether the Dl/N pathway acts as an inhibitor in bract specification we heat shocked pupae carrying *hs*-GAL4/UAS-*Nintra*, a constitutively active version of the N receptor (Rebay et al., 1993; Struhl et al., 1993). Heat shock, given at the stage at which previous results suggested bracts were specified (8-12 hours APF), had no effect on bristle pattern, but a high number of bristles lacked bracts (Fig. 4D). Heat shock at earlier stages strongly reduced the number of bristles, and with heat shock at later stages many bristles lacked both shaft and bract. These results suggest that earlier than 8 hours APF, the Dl/N signalling pathway is active in mediating lateral inhibition to specify the pattern of bristles. From approximately 8 hours APF, N signalling is silenced and the RAS/MAPK pathway activated by the SOP to activate bract fate in a neighbouring epidermal cell. Later, once bracts have already been specified, SOPs undergo the first division and Dl/N function is again required for the specification of the different components of the SOP lineage. We conclude that although the expression of an unidentified gene might define the spatial and temporal limits of cellular competence, the activity of Dl/N signalling also contributes to the restriction of the early and late limits of competence.

Only mechanosensory bristles induce bract fate

ChBs of the leg have no bracts. We consider two models to explain this: (1) ChBs are specified before the time of competence for bract induction, or (2) ChBs prevent bract specification by inhibiting Spi signalling.

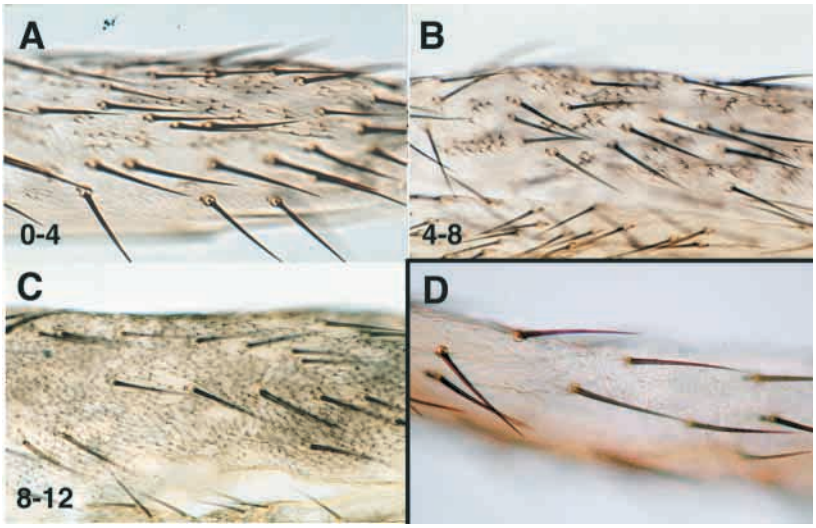

Fig. 4. The competence to specify bracts is temporally restricted. *hsp70*-GAL4/UAS-*Raf* * pupae heat shocked for 30 minutes, 37°C at 0-4 (A), 4-8 (B) or 8-12 (C) hours APF. Note that in B, the patches of bracts do not appear close to the bristles. (D) Notch activation represses the specification of bracts. *hsp70*-GAL4/UAS-*Nintra* pupae heat shocked for 30 minutes, 37°C at 8-12 hours APF. All bracts are missing.

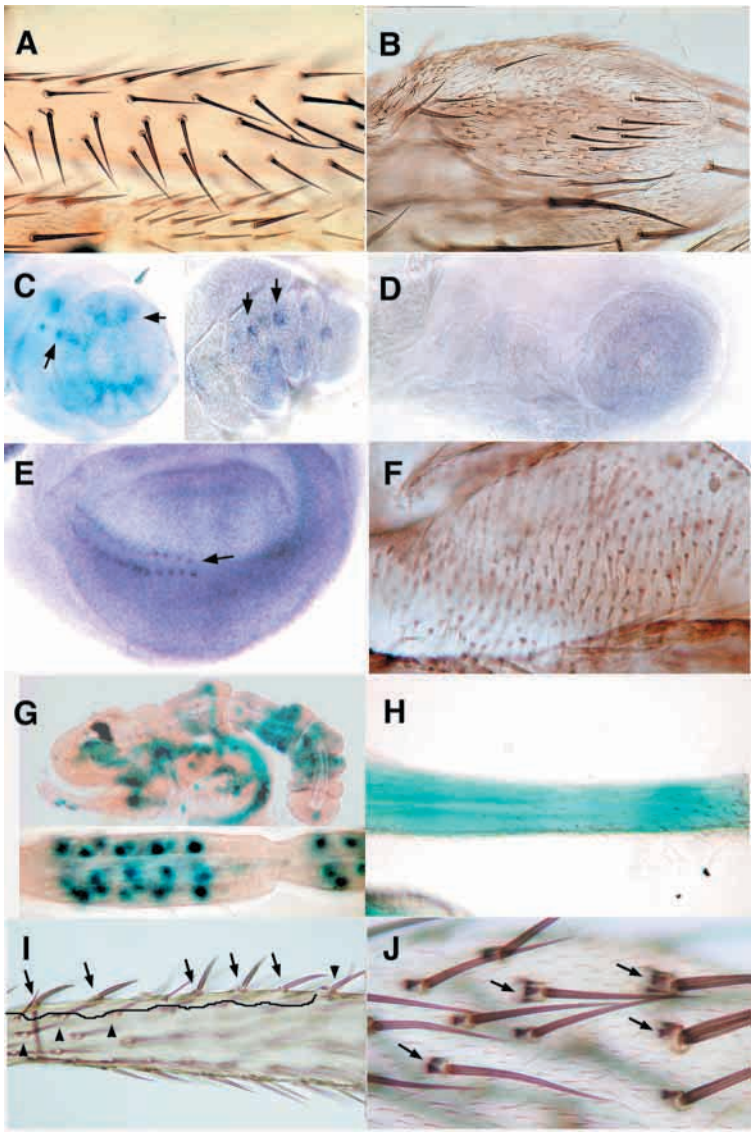

Fig. 5. (A) Effect of *poxn* overexpression induced by heat shock for 30 minutes, 37°C at 8 hours APF. In the leg, MBs are transformed into ChBs and bracts are missing. (B) Overexpression of *poxn* and *Raf** yields an additive phenotype, with trichomes transformed into bracts and MBs into ChBs. (C-E) *poxn* expression detected by *poxn* RNA *in situ* hybridisation in early pupal leg (C) and wing (E) of wild-type, and leg of *In(1)sc*$^{10-1}$ (D). (F) *hsp70*-GAL4/UAS-*sSpi* leg. All the trichomes are transformed into bracts. (G,H) *rho1*-lacZ expression in wild-type and *In(1)sc*$^{10-1}$ legs, respectively. (I) A *rho1*^{de11} clone marked with *f* is outlined. Note the absence of bracts within the clone (arrows) and the presence of bracts in the wild-type bristles that surround the clone (arrowheads). (J) UAS-rho1 and UAS-*S* overexpression by *hsp70*-GAL4. Note the presence of three bracts in some bristles (arrows).

The time for specification of ChBs in the leg has been mapped at 4 hours before puparium formation (BPF) (Rodríguez et al., 1990). This time is outside the period of competence for bract induction, which would support the first hypothesis as although chemosensory precursors were able to activate the pathway, epidermal cells would be unable to adopt the bract fate.

The gene *pox-neuro* (*poxn*) encodes a transcriptional regulator that acts as a selector gene for the specification of chemosensory fate (Bopp et al., 1989; Dambly-Chaudière et al., 1992). In *poxn* mutant flies, ChBs are either lost or transformed into MBs (Awasaki and Kimura, 2001) and the overexpression of *poxn* transforms MBs into ChBs (Nottebohm et al., 1992) (Fig. 5A). We heat shocked pupae carrying *hsp70-poxn* and *hsp70*-GAL4/UAS-*Raf**. In these flies MBs are transformed in ChBs, and all epidermal cells are transformed into bracts (Fig. 5B). These results suggest that *poxn* may operate in the SOP cell, and the RAS/MAPK pathway in the bract cell. We have looked at the expression of *poxn* in leg and wing discs of wandering larvae and early pupae and we found that *poxn* is expressed in single cells that probably correspond to chemosensory precursors (Fig. 5C, E). *poxn* is not expressed in *In(1)sc*$^{10-1}$ flies, which lack most of the leg bristles (Fig. 5D). Together, these results would support the second hypothesis as it seems *poxn* expression in SOP cells is sufficient to repress Spitz signalling.

spi is ubiquitously expressed in imaginal discs. The membrane-bound form of Spi needs to be cleaved into a soluble form to be active. To confirm that Spi is the only ligand that participates in this signalling, we have ubiquitously expressed a constitutively activated form of the Spi protein (*hsp70*-GAL4/UAS-*sSpi*) (Schweitzer et al., 1995). We obtained the same phenotype as those we obtained with overexpression of activated Raf, with the same temporal and spatial requirements (Fig. 5F). This result, together with *spi* clonal analysis (see above), indicates that in the developing leg, Spi signalling is necessary and sufficient to induce bract fate.

The overexpression of both Poxn and sSpi (*hsp70-poxn*, *hsp70*-GAL4/UAS-*sSpi*) shows the same phenotype as seen with overexpressed Poxn and Raf* (Fig. 5B); MBs are transformed in ChBs and trichomes are transformed into bracts. This confirms that Poxn must act in SOP cells by inhibiting Spi processing.

Spi cleavage requires two additional transmembrane proteins encoded by the genes *rhomboid1* (*rho1*) and *Star* (*S*) (Bier et al., 1990; Rutledge et al., 1992). It is thought that EGFr activation is controlled by the highly regulated pattern of *rho1* expression. We examined the expression of *rho1* and found that, in early pupae, it is expressed in a dynamic pattern ending in single cell expression (Fig. 5G). In *In(1)sc*$^{10-1}$ pupae, this pattern of expression is missing (Fig. 5H). We tested the requirement for *rho1* in the induction of bracts by making *rho1* mutant clones in the leg. These clones proliferate normally and differentiate bristles, but do not differentiate bracts; indicating that *rho1* is required for bract induction (Fig. 5I). To test whether *rho1* and *S* are sufficient for bract induction, we heat shocked flies carrying *hsp70*-GAL4/UAS-*rho1* + UAS-*S* at the time of bract induction. In this experiment, we did not find bracts far from bristles nor associated to ChBs, and only a few MBs had two or three bracts (Fig. 5J).

The role of the *argos* gene in defining the number and position of bracts

There are three aspects we want to consider here: the number

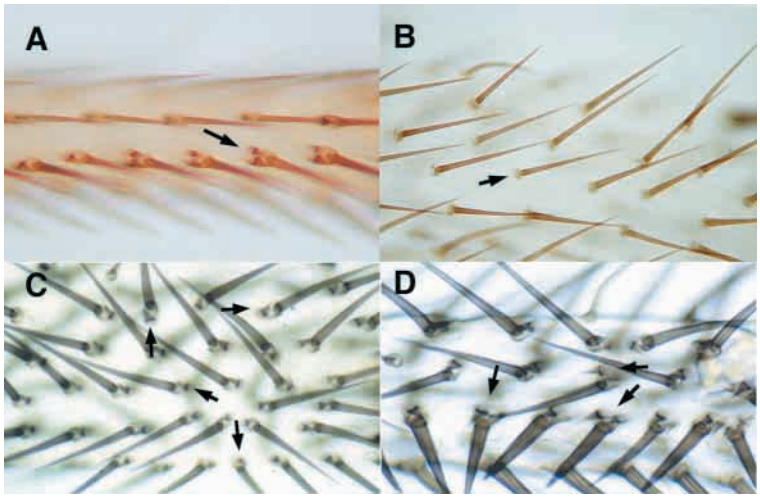

Fig. 6. Argos function is required to select which cell will become a bract. (A) *aos* loss-of-function produces extra bracts (arrow); $aos^{\Delta 15}$ leg. (B) Overexpression of *aos* removes all bracts (arrow); *sca*-GAL4/UAS-*aos*. (C) *sple* basitarsus showing mutant polarity. Arrows indicate sets of bracts and bristles pointing in different directions. (D) *sple*; *gap1* basitarsus; extra bracts show the same polarity and appear in the same position as endogenous bracts (arrows).

of bracts per bristle, bract polarity and the position of the bract. In wild-type flies, there is a single bract per bristle. This suggests a possible mechanism of lateral inhibition. One candidate mediator of this inhibition is the Dl/N signalling pathway. Nevertheless, pupae carrying the thermosensitive allele N^{ts}, heat shocked at different times during pupal development, do not have extra bracts (Held, 1990) (D. del Alamo, J. Terriente and F. J. Díaz-Benjumea, unpublished). We believe instead that the *argos* (*aos*) gene mediates this lateral inhibition. Flies homozygous for the viable allele $aos^{\Delta 15}$ show two or three bracts per bristle, clustered on its proximal side (Fig. 6A). Overexpression of Aos with *scabrous*-GAL4, which is expressed in proneural clusters, removes all the bracts (Fig. 6B). These results indicate that *aos* plays an active role in limiting the number of bracts per bristle.

Bract cells have the same polarity as the rest of the cells in the leg: trichomes, bristles and bracts all point distally. Mutations that affect the planar polarity of the tissue, such as *frizzled* (*fz*) or *spiny legs* (*spl*), also affect bract polarity (Fig. 6C). The double mutant combination *spl*; *gap1* shows extra bracts, all with the same polarity (Fig. 6D). This indicates a global mechanism for the establishment of polarity in leg discs.

A subject distinct from the polarity is the location of the bract relative to the bristle. Bracts are always located opposite to where the bristle polarity points. In wild-type legs, this location corresponds to the proximal-most side of the bristle. In three different situations in which the pathway is overactivated – *aos* mutants (Fig. 6A), *gap1* mutants (Fig. 2B) and *rho1* overexpression (Fig. 5J) – the extra-bracts appear clustered on the proximal-most side of the bristle. This suggests that the inductive signal is polarised to proximal. Therefore the position of the bract is likely to be determined by a polarised signal from the bristle. Nevertheless, we cannot reject other possibilities, such as a greater competence of cells at the proximal-most side to respond to the inductive signal, or that bracts are induced anywhere close to the bristle and later moved to the final position through differences in cell affinity.

DISCUSSION

Cells adopt different fates according to the cellular context in which they develop and to a great extent this influence is mediated by intercellular signalling. One of the main challenges in cell signalling is to identify how a signal is temporally and spatially regulated, and how signalling provokes specific responses in competent receiving cells. We have analysed the mechanism of the specification of bract fate in *Drosophila* legs. Previous results have shown bracts to be single epidermal cells that depend for their specification on the nearby bristle cell. We first confirmed that bristle and bract cells do not belong to the same lineage. We analysed the potential role of the RAS/MAPK signalling pathway in the determination of bracts by removing the activity of the pathway in clones of mutant cells, and by expressing a constitutively activated version of the pathway. We conclude that the RAS/MAPK pathway mediates an inductive signal, originating in the bristle cell, that establishes bract fate in a neighbouring epidermal cell. This signalling is mediated by the ligand encoded by the *spi* gene and the receptor encoded by the *Drosophila* EGFr homologue (Fig. 7).

Bract fate specification is controlled at three levels

How is bract fate specified? Our results indicate that the acquisition of bract fate is controlled at three levels. One level of control takes place in the receptor cell, where the competence to acquire bract fate is spatially and temporally controlled. Ubiquitous expression of activated Raf provided in short pulses of time indicated that the competence to acquire bract fate is spatially restricted to specific regions of imaginal discs. There is also a temporal restriction to early pupal development, with peak competence between 8-12 hours APF. These results are consistent with there being a temporally and spatially restricted expression of a tissue-specific regulator that gives the receptor cell the competence to activate bract fate (X in Fig. 7C).

This view is similar to that observed in the specification of bristles by the *AS-C* genes. Although the expression of *AS-C* genes is spatially and temporally restricted to cell clusters, ubiquitous *ac* and *sc* expression provided at different developmental times, in a genetic background that lacks the endogenous genes, results in a pattern of bristles of the correct type, located in the wild-type positions. So, both pattern and type of bristles are defined by the developmental context in which *AS-C* genes are expressed (Rodríguez et al., 1990).

We also observed that the Dl/N pathway represses bract fate specification. Overexpressing both Raf* and Nintra, we observed a strong reduction in the number of ectopic bracts produced by Raf* (data not shown). This result suggests that N signalling acts downstream of Raf in the inhibition of the RAS/MAPK pathway.

Another level of control occurs in the bristle cell that sends the inductive signal. Spi protein requires the functions of *rho1* and *S* genes to be processed into a soluble, activated form. *S*

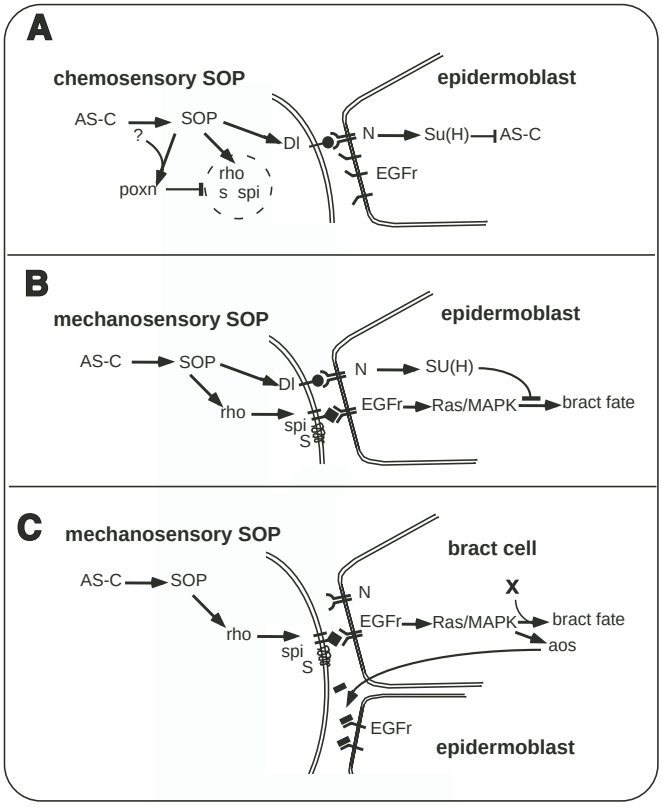

Fig. 7. A model of the specification of bract cells. (A) In chemosensory organ precursor cells, *poxn* expression represses the activation of Spi ligand, and this inhibits the specification of bract fate. (B,C) In mechanosensory organ precursor cells, Spi is activated and signals to a neighbouring cell to activate, through the EGFr, the RAS/MAPK pathway. In regions where cells are competent (expression of an unidentified gene 'X') the activation of the pathway drives the specification of the bract fate. This competence is temporally restricted. Thus, in early pupal development (B), epidermal cells may not be competent as cells do not differentiate as bracts after the activation of the pathway. The activation of the Dl/N signalling pathway, which mediates the lateral inhibition mechanism to specify a single SOP cell, also represses the specification of bracts by antagonising the RAS/MAPK pathway. (C) At 8 hours, APF bract cells are specified, and the activation of the *aos* gene in the bract cell inhibits the specification of more neighbouring cells as bracts.

and *spi* are ubiquitously expressed, and *rho1* is expressed in SOP cells. The phenotype of *rho1* mutant cells in clones indicates that *rho1* is required for the induction of bract fate. Nevertheless, *rho1* is also expressed in bract-less ChBs, and ubiquitous overexpression of *S* and *rho1* results in a mild phenotype of extra bracts in wild-type positions. Together these results suggest that another component, whose expression must be restricted to the SOP of MBs, is required for bract induction.

The expression of the *poxn* gene is both necessary and sufficient for the specification of bract-less ChBs. As ChBs are specified before epidermal cells are competent for bract induction this provides an explanation for the bract-less phenotype of ChBs. Nevertheless, Poxn overexpression suppresses bracts, and the result of the combined overexpression of Poxn and activated Raf or sSpi indicates that Poxn acts in the SOP cells to repress Spi signalling. Poxn and Rho1 are co-expressed in the SOP. So these results do not allow us to deduce the molecular mechanisms by which poxn expression represses Spi signalling. Nevertheless, as the result of S and rho overexpression indicates that they are not sufficient to induce bracts, and that at least one other component present in the SOP cell is required, it can be tentatively suggested that poxn may act upstream of this other gene.

Bract number and position

Bracts always appear on the proximal-most side of the bristle, which raises the question of whether the position of the bract is defined by a polarised signal. Several lines of evidence suggest that the SOP cell is polarised (Hartenstein and Posakony, 1989; Huang et al., 1991). We have found that in all the experiments that result in extra bracts, these appear clustered on the proximal-most side. It seems likely that the polarisation of the SOP cell leads to a polarisation of Spi signalling which results in a constant position of the bract, although we cannot reject other possibilities.

Concerning the number of bracts per bristle, our results indicate that the *aos* gene plays a role in mediating a lateral inhibition mechanism. Loss of *aos* led to clusters of bracts, while overexpression removed all bracts. It is reasonable to think, therefore, that as a result of the activation of the RAS/MAPK pathway in the presumptive bract cell, *aos* expression is being activated in this cell to inhibit the pathway in neighbouring epidermal cells.

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