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Hedgehog and Beyond

Minireview

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One goal of developmental biology is to elucidate the mechanisms by which signaling molecules establish positional information within a field of cells. In the past few years, a number of secreted signaling molecules, including members of the transforming growth factor β (TGFβ), Wnt, fibroblast growth factor (FGF), and hedgehog (hh) families, have been implicated in patterning of several different structures in Drosophila and vertebrates. A number of recent experiments have furthered our understanding of the mechanisms by which these molecules control body patterning. Hh has been shown to control the expression of TGFβ, Wnt, and FGF signaling molecules, leading to models pertaining to the regulatory interactions among these inductive signals. Biochemical analyses have shown that the hh protein undergoes autoproteolysis, generating two peptides, each with distinct biochemical properties, and possibly providing the basis for the short- and long-range signaling effects of hh. Finally, hh was found to regulate the expression of secondary signals by counteracting the activity of a cAMP-dependent protein kinase (PKA) regulatory system, linking hh with a previously known signal transducer.

Hh Initiates Both Short-Range and Long-Range Signaling

An unusual property of the secreted hh proteins is that they are associated with both short-range and long-range actions, depending on their developmental context (reviewed by Smith, 1994). An example of short-range action of hh in Drosophila is found in the ventral epidermis, where hh causes adjacent cells to maintain *wingless* (*wg*) expression (reviewed by Perrimon, 1994). Similarly, in the vertebrate central nervous system, Sonic hedgehog (Shh) is expressed in notochord cells and induces floor plate formation within the adjacent neural tube in a contact-dependent manner (Roelink et al., 1994).

In addition to these short-range actions, hh is also associated with long-range effects, which appear to arise both directly and by activating secondary subordinate signaling molecules. Recent studies of the precursors of Drosophila adult structures have clearly established that one of the mechanisms by which hh organizes pattern is by directing the expression of secondary signals. Each adult appendage is comprised of cuticular structures that are derived from groups of larval imaginal cells. Appendage precursors are subdivided into anterior and posterior compartments and express posteriorly the homeodomain protein engrailed (en) as well as hh (Figure 1A). In the anterior compartment of leg imaginal discs, two signals, wg and the TGFβ family member decapentaplegic (dpp), are expressed in distinct patterns. *wg* expression is confined to a ventral sector while *dpp* is expressed dorsally along the anteroposterior (AP) border. Analysis of the leg phenotypes associated with loss-of-function *hh* mutations and ectopic expression of hh has led to the proposal that posterior cells organize growth and cell patterning in both compartments by secreting hh (Basler and Struhl, 1994; Diaz-Benjumea et al., 1994; Capdevila et al., 1994; Tabata and Kornberg, 1994). Loss of hh activity at the AP compartment boundary is associated with loss of *dpp* and *wg* expression and with abnormal cellular proliferation and patterning. Ectopic expression of hh in the anterior compartment causes dramatic reorganizations of the anterior compartment pattern and is associated with an expanded expression of *wg* ventrally and ectopic expression of *dpp* dorsally. Thus, in the leg disc, secretion of hh into the anterior compartment activates *wg* ventrally and *dpp* dorsally. It is presumed that subsequent control of growth and cell patterning is achieved by activation of the wg and dpp signaling pathways. In addition, cooperation between inductive signals is required to trigger formation of the proximodistal axis. Cells in the center of the leg disc that receive wg and dpp signals express genes such as *aristaless* (*al*) and *Distal-less* (*Dll*), which are required for proximodistal axis formation (Campbell et al., 1993; Diaz-Benjumea et al., 1994).

Long-range effects of hh may also arise directly, and two examples have been reported. In the Drosophila dorsal epidermis, four different cell types, referred to as 1°, 2°, 3°, and 4°, can be identified. Hh, which is expressed in the 1° cells, appears to function as a diffusible morphogen to instruct neighboring 2° cell types and more distant 3°

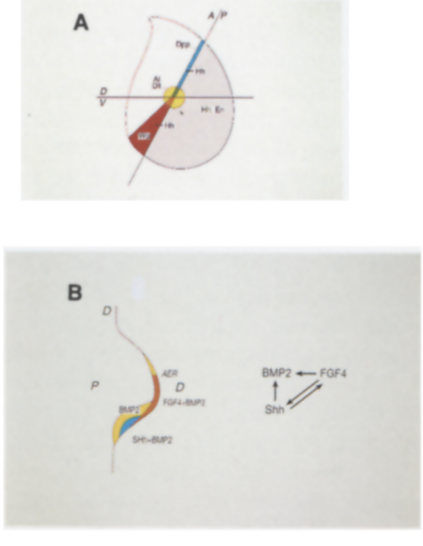

Figure 1. Spatial Expression and Regulatory Interactions between *hh* and Other Genes in the Leg Imaginal Disc and the Developing Vertebrate Limb Bud

(A) Leg imaginal disc. A, anterior; D, dorsal; P, posterior; V, ventral.
(B) Developing vertebrate limb bud. D, distal; P, proximal.

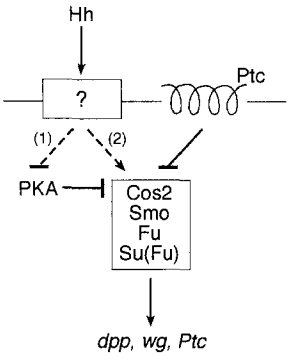

Figure 2. Putative Interactions among Members of the Hh Signaling Pathway

Models favored by Li et al. (1995) (1) and Jiang and Struhl (1995) (2). The position of fu, Su(fu), smo, Cos^2, and ci as targets of PKA is speculative and simply indicates that the interactions between these genes and PKA need to be examined.

cell types in a concentration-dependent fashion (Heemskerk and DiNardo, 1994). A low level of hh induces 3° cell fates without inducing 1° or 2° cell fates, whereas higher levels induce 2° cell fates while suppressing 3° and 4° cell fates. Similarly, studies on the long-range induction of sclerotome by the notochord have provided evidence for a direct long-range action of Shh. Fan and Tessier-Lavigne (1994) have shown that both the notochord and COS cells expressing Shh can induce the expression of the sclerotomal marker *Pax1* in presomitic mesoderm explants over hundreds of micrometers and across nucleopore filters. Furthermore, they showed that *Pax1*-expressing mesoderm does not itself induce *Pax1* expression in these explants, suggesting that the long-range effect does not result from a cascade of short-range interactions and supporting a direct long-range action of Shh.

Feedback Loops

One mechanism by which hh proteins organize tissue pattern is by regulating the expression of signals in adjacent cells. In some instances, hh-receiving cells signal back to the hh-secreting cells, thus providing a means to coordinate the expression of inductive signals with pattern formation. Two such examples are found in the Drosophila ventral epidermis and morphogenesis of the vertebrate limb bud.

In studies in the Drosophila embryo, a connection between hh and another signal, the secreted glycoprotein wg, has demonstrated an intimate regulatory loop between the expression of these two signals (reviewed by Perrimon, 1994). During embryonic segmentation, *wg* and *hh* are expressed on either side of the metameric unit. The maintenance of their expression is critical to patterning since in the absence of either gene function, segmentation does not occur. Intricate interactions between *wg*-expressing cells and *hh*-expressing cells are revealed by the observation that continued *wg* expression requires hh activity and vice versa. In addition, ectopic expression of hh is associated with an expansion of the *wg* domain. The interdependency of the regulation of *hh* and *wg* demonstrates the existence of regulatory loops that act to ensure the coordinated expression of inductive signals during the patterning of a field of cells.

Similarly, studies in the developing vertebrate limb bud have revealed that Shh, like Drosophila hh, induces expression of secondary signals, including the TGFβ-like protein BMP2 in the mesoderm and FGF4 in the ectoderm (Laufer et al., 1994; Niswander et al., 1994; Figure 1B). In the developing limb, two signaling centers, the zone of polarizing activity (ZPA) and the apical ectodermal ridge (AER), organize pattern and growth of the developing limb. Shh has been proposed to encode the endogenous ZPA signal since it has polarizing activity and its expression colocalizes with ZPA activity. Similarly, *FGF4* has been proposed to encode the endogenous AER signal since it localizes to the posterior ridge and can replace the activity of the ridge. Analyses of the regulatory interactions between Shh and FGF4 have revealed the existence of feedback loops that control the expression of these signals. Expression of *FGF4* in the AER can be induced by Shh. However, despite the fact that FGF4 cannot induce *Shh* expression, it is required to maintain its expression. These results reveal a positive feedback loop, initiated by Shh, which maintains the expression of Shh in the posterior mesoderm and FGF4 in the AER. In addition, cooperation between the Shh and FGF4 signals is required to control the expression patterns of genes such as *BMP2* in the mesoderm, indicating that the ability of cells to respond to Shh is dependent on FGF4 proteins produced by the AER.

There May Be Two Distinct Hh Signaling Pathways

Another level of complexity in understanding hh signaling arose from biochemical studies of the hh protein (Lee et al., 1994). Studies in Drosophila have shown that *hh* encodes a 46 kDa native protein that is first cleaved into a 39 kDa form following signal sequence cleavage and subsequently into two major forms: a 19 kDa amino-terminal form (hh-N) and a 26 kDa carboxy-terminal form (hh-C). Vertebrate hh proteins appear to be processed in a manner similar to the Drosophila proteins (Lee et al., 1994; Chang et al., 1994; Bumcrot et al., 1995). Interestingly, hh itself catalyzes the intramolecular cleavage that generate hh-N and hh-C. When analyzing the characteristics of hh-N and hh-C, Lee et al. (1994) found that the two proteins have different biochemical properties and are differentially distributed. Experiments using Drosophila S2 cells reveal that hh-N remains associated with the cell pellet, while hh-C is released into the supernatant. In addition, hh-N, but not hh-C, is recovered bound to heparin–agarose beads, a characteristic of proteins that associate with the extracellular matrix. Consistent with these findings is their distribution in embryos where the hh-N expression pattern is reminiscent of the *hh* transcripts, while hh-C has a much broader distribution, indicating that it is able to diffuse. The exact distance each protein form can travel is not yet resolved, although results with hh-N suggest that it may not diffuse and thus may only signal to immediately adjacent cells.

Autoproteolysis appears to be important for hh function. Lee et al. (1994) used an in vivo overexpression assay to compare the patterning effects of wild-type hh and a mu-

tant form of hh, H329A, which is unable to undergo proteolysis. They compared the activities of these two proteins in different tissues where hh has been shown to act either as a short-range or long-range signal. In the ventral embryonic epidermis, hh acts as a short-range signal to maintain *wg* expression in adjacent cells, and overexpression of hh is associated with an expansion of the domain of *wg* expression. Ectopic expression of H329A also led to an expansion of *wg*, although the expansion was not as extensive as observed with overexpression of wild-type hh. In the dorsal embryonic epidermis, hh acts as a long-range signal. Unlike wild-type hh, overexpression of H329A in the dorsal epidermis does not alter epidermal fates, suggesting that cleavage of hh into hh-N and hh-C is required for this effect. These results demonstrate that autoproteolysis is important for full hh activity but also raises the possibility that hh-N is responsible for the short-range signaling effects while hh-C is involved in the long-range actions of hh (Lee et al., 1994). This model implies that, since the hh-N and hh-C sequences are different, hh may activate two different signaling pathways. A definitive test of this model will require an analysis of the function of each hh form individually.

The Role of PKA in Hh Signaling

The biochemical mechanisms by which hh proteins regulate the activities, transcription, or both of secondary signal transducers is poorly understood. A number of gene products, including the multitransmembrane protein patched (ptc), the transcription factor cubitus interruptus (ci), the serine/threonine kinase fused (fu), and the as-yet-unidentified gene products of *Costal-2* (*Cos*^{2}), *smoothened* (*smo*; initially named *smooth* by Nüsslein-Volhard et al., 1984), and *Suppressor of fused (Su(fu))*, have previously been implicated as putative components of the hh signaling pathway (Forbes et al., 1993; Hooper, 1994; Perrimon, 1994). As reported in this issue of *Cell* and in *Nature*, the serine/threonine cAMP-dependent protein kinase PKA is also involved in the mechanism by which hh operates.

PKA consists of two catalytic and two regulatory subunits. In response to extracellular stimuli, cAMP binds to the regulatory subunits that release the catalytic subunits. The activated subunits then regulate the activity of other molecules by phosphorylation. With the exception of its role during Dictyostelium development (Devreotes, 1994), little is known about the function of PKA during development. In this issue of *Cell* and in *Nature*, Pan and Rubin (1995), Li et al. (1995), Jiang and Struhl (1995), Lepage et al. (1995), and Strutt et al. (1995) describe the phenotype associated with clones of imaginal disc cells homozygous for a null mutation in the catalytic subunit of PKA. In imaginal discs destined to form appendages, *Pka*$^-$ clones induced within the anterior compartment are associated with dramatic pattern duplications while clones induced within the posterior compartment or at the AP border have no apparent effect on disc patterning. The pattern alterations extend beyond the boundaries of the *Pka*$^-$ clones and affect the growth and fate of neighboring cells. The observed pattern respecifications associated with loss of PKA activity are similar to those generated following ectopic expression of hh. Therefore, the authors examined the expression of hh target genes such as *dpp* and *wg* in clones of *Pka*$^-$ cells. In the leg imaginal disc, clones of *Pka*$^-$ cells turn on *dpp* in the dorsal–anterior quadrant of the disc and *wg* in the ventral–anterior quadrant. Thus, cell fate changes associated with *Pka*$^-$ clones, identical to those generated by ectopic expression of hh, can be attributed to the ectopic expression of the subordinate signals. For example, in the anterior compartment of the wing discs, where loss of PKA activity turns on *dpp*, clones of cells doubly mutant for *Pka* and *dpp* are not associated with pattern duplications. The similarity of phenotypes generated by loss of PKA activity and ectopic expression of hh extends to processes other than appendage pattern specification. Of particular interest is the situation in the eye disc, where hh controls the movement of the morphogenetic furrow (MF) from posterior to anterior. Cells anterior to the MF divide and are unpatterned while cells posterior to the MF differentiate into ommatidial clusters. In wild type, hh is expressed posterior to the MF and activates *dpp* within the MF. Clones of *Pka*$^-$ cells anterior to the MF behave as do cells within the MF; i.e., they express *dpp*, as well as other MF-specific markers, and they differentiate prematurely (Pan and Rubin, 1995; Strutt et al., 1995).

What is the relationship between hh and PKA? The possibility that loss of PKA activity turns on *hh* can be ruled out since no ectopic *hh* expression is seen in *Pka*$^-$ clones. Furthermore, clones of *Pka*$^-$ cells maintain their patterning abilities when induced in a genetic background where hh expression is reduced, indicating that hh is not required for transcriptional induction of *dpp* in clones of *Pka*$^-$ cells. The genetic observation that a dominant-negative allele of PKA acts as a dominant suppressor of a partial loss-of-function *hh* mutation (Pan and Rubin, 1995) suggests that hh and PKA operate in the same developmental process. However, whether hh directly regulates PKA expression is a matter of controversy. Two mechanisms by which hh represses the effect of PKA can be imagined (Figure 2). In the first, hh and PKA act in the same linear biochemical pathway, with hh relieving the PKA-mediated repression of downstream target genes. In the second, hh and PKA act antagonistically in two parallel pathways. Both Li et al. (1995) and Jiang and Struhl (1995) have addressed this question by asking whether a constitutively active form of PKA can block the effect of hh. Li et al. found that constitutively active PKA can counteract hh function at the AP border while, in contrast, Jiang and Struhl observed no such effects. Because both studies used different techniques to drive the misexpression of constitutively active PKA, it is possible that higher levels of constitutively active PKA were expressed in the experiments by Li et al. than in those by Jiang and Struhl. If this is the case, then results from both analyses may simply reflect the competition between an activating (hh) and a repressing (PKA) regulatory system that converges on some common downstream targets. This issue needs to be clarified in future studies in order to resolve whether hh regulates PKA.

Does Ptc Regulate PKA Activity?

Studies in Drosophila have led to the hypothesis that ptc is a hh receptor (Ingham et al., 1991; reviewed by Perrimon, 1994). According to this model, ptc would act as a repres-

sor of *wg* transcription in the embryo, with only those cells receiving the hh signal able to overcome this repression. However, this hypothesis has become clouded by the finding that tissues exist in which the requirements for ptc and hh can be dissociated. One such example is the dorsal embryonic epidermis, where ptc is not expressed in cells whose fates can be specified by hh (Heemskerk and DiNardo, 1994). In addition, ptc has been found to be associated with hh-independent function in both the embryo and imaginal precursors (Bejsovec and Wieschaus, 1993; Capdevila et al., 1994). Thus, the role of ptc in hh signaling is unclear, and it is possible that hh and ptc act antagonistically in two parallel pathways.

Analysis of the relationship between ptc and PKA provides additional insights about the role of ptc in hh signaling (Pan and Rubin, 1995; Li et al., 1995; Jiang and Struhl, 1995; Lepage et al., 1995). In discs destined to form appendages, ptc is expressed at low levels in the anterior compartment and at high levels along the AP border (Tabata and Kornberg, 1994; Capdevila et al., 1994). Expression of ptc along the AP border is regulated by hh, PKA, and ptc itself. In the absence of hh, ptc represses its own activity. Like ectopic hh expression, loss of PKA activity in the anterior compartment or at the AP border is sufficient to promote *ptc* expression. Expression of a constitutively active form of PKA at the AP border can repress *ptc*, as can the reduction of hh activity in the posterior compartment. Ptc does not appear to regulate PKA activity since a constitutively active form of PKA is not able to substitute for loss of ptc activity in clones of ptc^- cells. In addition, ubiquitous overexpression of ptc is unable to affect the overall level of PKA activity, although it is effective in reducing ptc expression at the AP border. Together, these observations suggest that ptc itself does not regulate PKA activity (Figure 2).

Concluding Remarks

Studies on hh have illustrated that to understand the mechanism by which an inductive signal organizes pattern, one has to know whether a signal directly regulates gene expression in cells to which the signal binds or whether it acts indirectly through a cascade of subordinate signals. The elucidation of the mechanism can be difficult as it requires not only the identification of target genes that respond directly to the signal, but also knowledge of the distribution and biochemical properties of the signal. Finally, interpretation of the results of experiments designed to test the instructive properties of the signal can be further complicated if the expression of the signal is regulated by feedback loops originating from the cells that receive the signal.

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