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Shaping Morphogen Gradients

Minireview

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The proper functioning of tissues and organs requires that each cell differentiate appropriately for its position. In many cases, the positional information that instructs cells about their prospective fate is conveyed by a morphogen gradient. Morphogens are signaling molecules that are produced in a restricted region of a tissue and move away from their source to form a long-range concentration gradient. Cells differentiate in response to morphogen signaling depending on their position within the gradient and thus on their distance from the morphogen source. Two criteria have gained acceptance as the evidence needed to qualify a secreted signaling protein as a morphogen: concentration dependence and direct action at a distance. The Wingless, Hedgehog (Hh), and Dpp gradients in the *Drosophila* wing and leg imaginal discs fulfill both criteria (for literature, see Teleman and Cohen, 2000). First, multiple target genes are induced at different ligand concentrations, with low threshold responses elicited in cells at a greater distance from the source. The second criterion requires a demonstration that the morphogen acts directly on responsive cells, and that it does not act at a distance by relay of a secondary signal. An example of signal relay is Dpp in the fly wing. Hh acts directly over 8–10 cells to induce Dpp, which in turn acts directly on distant cells in the form of a long-range symmetric Dpp gradient. There is abundant evidence for concentration-dependent activity of signaling proteins in vertebrate development (reviewed in Gurdon et al., 1998). However, evidence for direct action at a distance has been lacking. Two new papers address this issue (Chen and Schier, 2001; Briscoe et al., 2001).

Sonic Hedgehog (Shh) acts in a concentration-dependent manner to define domains of gene expression and specify neuronal cell fates in the developing central nervous system (Briscoe et al., 2000; Agarwala et al., 2001). In a study soon to be published, Briscoe et al. (2001) have made an important step forward in demonstrating that Shh acts directly at a distance to pattern the neural tube. They have made use of a mutant form of the Sonic hedgehog receptor Patched1 to produce cells that are not responsive to Shh. The design of these experiments is based on the regulatory relationship between the two components of the Shh receptor, Patched and Smoothened. Smoothened is required to transduce the Shh signal. Smoothened activity is held in check by Patched. Chen and Struhl (1996) have previously shown that Hh binding to Patched alleviates the block to Smoothened activity and allows transduction of the Hh signal. Briscoe et al. made use of a mutant form of Patched1 that retains the ability to inactivate Smoothened, but that cannot bind Shh. When the mutant Patched1 protein was randomly expressed in the chick neural tube using electroporation, they observed cell autonomous repression of Shh target genes. Repression was observed for target genes that are induced in response to different levels of Shh activity and at different distances from the source. These observations provide unequivocal evidence that Shh acts directly on distant cells and therefore that Shh forms a morphogen gradient in the developing neural tube.

Evidence for another bona fide vertebrate morphogen has just been reported by Chen and Schier (2001). They performed an elegant series of experiments showing that the Nodal-related TGF-β protein Squint acts both in a concentration-dependent manner and directly at a distance in the Zebrafish embryo. They injected single cells in embryos with Squint mRNA and showed that target genes were activated in both nearby and distant cells. High threshold genes were activated only near the Squint source, whereas low threshold genes were also activated in more distant cells. Reducing the amount of mRNA injected resulted in only low-threshold genes being activated, showing concentration-dependent action of Squint. To prove direct action at a distance, they used embryos mutant for Oep, a cofactor required for cells to respond to Squint signaling. By injecting a single cell of an Oep mutant embryo with Squint mRNA and implanting at a distance a wild-type cell that could respond to Squint signaling, they proved no relay mechanism is required, indicating that Squint functions as a morphogen.

Morphogen gradients have recently been visualized directly by antibody staining or using morphogen-GFP fusion proteins that retain signaling activity (Entchev et al., 2000; Incardona et al., 2000; Strigini and Cohen, 2000; Teleman and Cohen, 2000; Lewis et al., 2001 [this issue of *Cell*]). In spite of considerable interest in morphogen gradients over the last few years, the problem of how they form in tissues has not been fully explored. Gradients could form passively by diffusion of soluble protein or by facilitated diffusion of morphogen bound to a carrier. As alternatives, mechanisms for active transport of morphogens have been postulated. One possibility involves successive rounds of internalization of the ligand by endocytosis followed by resecretion. This would serve to pass the molecules from cell to cell and facilitate movement across the tissue (Figure 1). A second possibility involves long cellular processes that project toward the localized morphogen source. In the following sections, we will discuss the evidence for active transport and then consider how tissues might make use of passive processes to shape morphogen gradients.

Do Ligands Move by Vesicle-Mediated Transport?

Ideas about vesicle-mediated transport have their roots in the observation that almost all morphogens have been seen in intracellular vesicles. Repeated cycles of endo-

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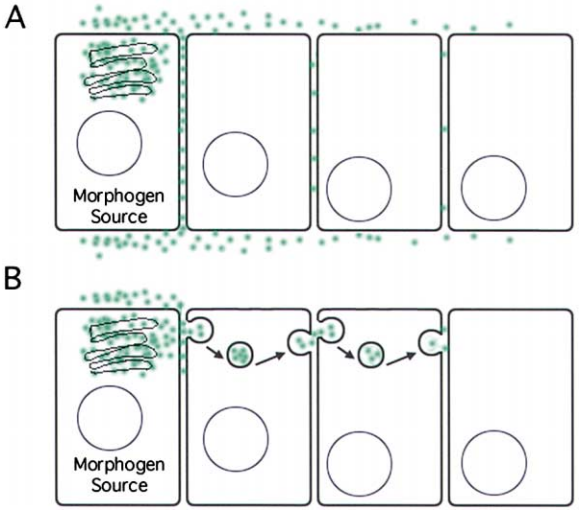

Figure 1. Models for Morphogen Movement through Tissues

A morphogen may move (A) solely by extracellular diffusion or (B) solely by repeated rounds of endocytosis and resecretion. The truth may lie somewhere between these extremes.

cytosis and resecretion, could, in principle, move ligands through tissue. The strongest evidence in support of a role for endocytosis in ligand transport comes from analysis of Dpp gradient formation in the *Drosophila* wing imaginal disc. Using biologically-active Dpp-GFP, Entchev et al. (2000) have shown that clones of cells unable to endocytose Dpp-GFP present a barrier to ligand movement. These experiments were done using mutants in the *Drosophila* gene encoding Dynamin, a GTPase required for clathrin-dependent endocytosis. Dynamin mutant clones can "cast a shadow" in the Dpp-GFP gradient. In other words, the level of Dpp-GFP was lower in cells located on the far side of the clone from the Dpp-GFP source than in nearby cells that were not "behind" the clone. This was only observed when Dynamin mutant clones were challenged with a wave of newly synthesized Dpp-GFP, but not under steady-state conditions. To compare dynamic and steady state conditions, we have generated a simulation to model the behavior of a ligand gradient when confronted by a zone through which the ligand cannot move. The simulation predicts that the clone would cast a shadow under both transient and steady-state conditions. However, the relative magnitude of the decrease in ligand levels is less at steady-state, so this would be more difficult to observe experimentally. The model also predicts more rapid accumulation of ligand in front of the clone (see supplemental movie and the corresponding legend for more detailed discussion: http://www.cell.com/cgi/content/full/105/5/559/DC1). It will be interesting to see whether careful measurement of ligand levels agrees with these predictions.

Although these results suggest a role for endocytosis in Dpp movement, at least part of the movement of Dpp does appear to take place extracellularly. Removing the Dpp receptor, Thickveins, in clones of cells, leads to accumulation of Dpp-GFP on the surfaces of the first few rows of cells on the side of the clone closest to the source of ligand (Entchev et al., 2000). If Dpp-GFP moved only by endocytosis, we might expect it to stop at the first mutant cell.

In contrast to Dpp, most Wg movement seems to occur extracellularly in the wing disc. Three lines of evidence support this view. First, Wg protein accumulates on the surface of cells expressing the Wg receptor DFz2 (Cadigan et al., 1998). Second, Wg can be visualized as an extracellular protein gradient by antibody staining, and third, Dynamin-mediated endocytosis does not appear to be required for formation of the extracellular Wg gradient (Strigini and Cohen, 2000). Instead, Wg protein accumulates to higher than normal levels in a long-range gradient on the surface of Dynamin mutant cells. Since secreted Wg is unstable, it must have moved across Dynamin mutant cells in order to accumulate on them. Thus, Dynamin-mediated endocytosis is not required for Wg movement in the wing disc.

In the *Drosophila* embryo, however, endocytosis has been proposed to mediate Wg movement. Dynamin mutants have been shown to reduce the range of Wg activity in the embryonic epidermis (Moline et al., 1999). This has been attributed to a defect in Wg transport, but could also reflect reduced Wg secretion as described in the wing disc (Strigini and Cohen, 2000). The best evidence for a role of endocytosis in Wg movement comes from experiments in which a dominant-negative form of Dynamin was expressed in the *engrailed*-expressing cells, posteriorly adjacent to the Wg-producing cells (Moline et al., 1999). Under these conditions, Wg secretion cannot be directly affected. Nonetheless, signaling to more posterior cells was reduced, suggesting a decrease in long range signaling. The differences between the wing disc and the embryo data are intriguing. Additional work will be required to determine whether Wg moves by different mechanisms in these tissues.

Endocytosis and Ligand Turnover

The idea that ligands might be moved by repeated cycles of endocytosis and secretion poses an interesting trafficking problem to the cell. How do cells distinguish between endocytic vesicles containing ligands targeted for degradation in the lysosome and those that might be recycled into the secretory pathway for secretion? Receptor-mediated endocytosis targets Shh and its receptor Patched for degradation (e.g., Incardona et al., 2000). As noted above, endocytosis removes Wg from the extracellular space in the wing imaginal disc. Both the range and shape of the Wg gradient are altered in discs where endocytosis is blocked using the Dynamin mutant (Strigini and Cohen, 2000). In this issue of *Cell*, Dubois et al. (2001) solve a long-standing mystery with their report that regulated degradation shapes the Wg gradient in the *Drosophila* embryo. Wg specifies whether embryonic ectodermal cells produce "naked" cuticle or cuticle with denticles. The puzzle has been how Wg could act over a much shorter range toward posterior than toward anterior. Although the Wg protein gradient is initially symmetric, it soon becomes asymmetric. Much less Wg protein is detectable in cells posterior to the Wg-producing cells than in anterior cells. Dubois et al. show that this difference is due to more rapid degradation of Wg in posterior cells. The exciting feature of this system is that the rate of degradation is both temporally

and spatially regulated in the tissue. Cells posterior to the Wg stripe are instructed to degrade Wg more rapidly by signals through the EGF receptor. These findings highlight the importance of ligand turnover in gradient formation. The range and slope of a morphogen gradient is determined by the rate of synthesis and by the rate at which the ligand is removed from the tissue.

Cytonemes

Another means by which active morphogen transport could be accomplished has been suggested by the observation that cells extend long cytoplasmic projections called "cytonemes" toward a morphogen source (Ramirez-Weber and Kornberg, 1999). Cytonemes could, in theory, transport Dpp or Wg away from their sources toward distant ligand-responsive cells. Although this possibility is tantalizing, no data in support of this mechanism has yet been reported. A challenge for the cytoneme model comes from the observation that ligand movement appears to be nondirectional in imaginal discs. Small groups of cells that ectopically express any of the three morphogens show symmetric activation of target genes around the source. In other words, there is no intrinsic orientation to the direction in which a gradient forms around an ectopic source. In contrast, cytonemes have an intrinsic orientation with respect to the axis that the morphogen normally patterns. For example, they extend from lateral to medial along the AP axis of the wing disc, oriented along the Dpp gradient. This appears to pose a problem, unless we are prepared to invoke a means of long-range communication that allows the cytonemes to identify the location of the new signaling center and to reorient toward it. It is also worth noting that cytonemes are found on the apical surface of the epithelium, whereas Dpp and Wg appear to travel on the basolateral side of the epithelium (Strigini and Cohen, 2000; Teleman and Cohen, 2000). Assessing the role of cytoplasmic projections in gradient formation will pose an interesting challenge for future work.

Limiting Ligand Movement through the Extracellular Space

While it is not yet clear that cells actively transport morphogens, it is clear that they actively limit their movement. In the case of Hedgehog (Hh) and Shh, this is accomplished in part by modifying the extracellular environment and in part by posttranslational modification of Shh/Hh. The signaling form of Shh/Hh is produced by endoproteolytic cleavage that leads to addition of a cholesterol moiety to the C terminus of the protein (for literature, see Lewis et al. 2001 [this issue of *Cell*]). Cholesterol-modified Hh or Shh bind to Patched and undergo receptor-mediated endocytosis (Burke et al., 1999; Incardona, et al. 2000). If Patched expression is reduced, cholesterol-modified Hh can move over longer distances, indicating a role for Patched in limiting the range of Hh movement (Chen and Struhl, 1996).

Lewis et al. now present evidence that cholesterol modification may be required to allow long-range activity of Shh in the mouse limb. Interestingly, Shh modification is not required for high-threshold responses in cells closest to the source, but appears to be required for lower-threshold responses in more distant cells. It is possible that cholesterol modification may be required for movement of Shh to form a long-range gradient. This is intriguing because earlier work in *Drosophila* suggested that unmodified Hh acts over a greater range than the cholesterol-modified form (Burke et al., 1999, and references therein). This resembles what happens to modified Hh when Patched levels are reduced. The difference in these two sets of results might reflect differences in the distances the gradients have to cover (300 μm in the mouse limb bud compared with up to 50 μm in the anterior compartment of the wing disc). Alternatively, it might reflect differences in the way the experiments were done (see Lewis et al. in this issue of *Cell* for more extensive discussion).

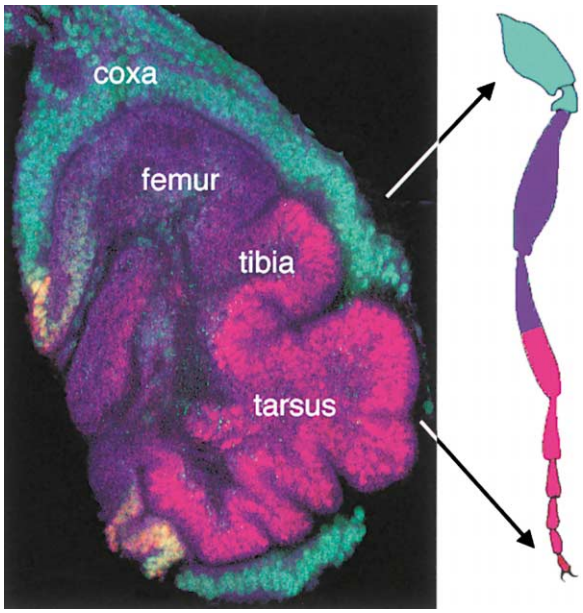

Figure 2. A *Drosophila* Leg Disc

Fluorescence micrograph of a *Drosophila* leg imaginal disc, labeled to visualize proteins that reflect regionalization of the leg along the proximal-distal axis. Distal-less is shown in red. Homothorax is shown in green. The whole epithelium is labeled in blue. The imaginal disc is a highly folded epithelial sheet. This causes cells that will be very proximal and cells that will be very distal in the adult to be near each other in the disc (illustrated at right). We thank Jun Wu for the disc picture.

At present, it is not clear how cholesterol-modified Shh/Hh actually moves through the tissue. Possibilities include carrier proteins that displace the cholesterol and palmitoyl groups from the membrane or shedding of exovescicles containing Shh/Hh from cells. In this context, it is interesting that a Patched-related protein, called Dispatched, is required in Hh-producing cells to release cholesterol-modified Hh (Burke, et al. 1999). Whether other proteins are implicated in movement after Hh gets to the surface of the Hh-producing cell remains to be determined.

Tissues also modulate Hh spreading by modifying the extracellular environment. An enzyme involved in heparan sulfate proteoglycan (HSPG) biosynthesis, known as Tout velu, is required for effective Hh gradient formation in the responding tissue (The et al., 1999). Hh levels are very low in patches of mutant tissue lacking Tout velu activity, presumably due to either reduced movement or reduced retention of Hh. In a comparable manner, the extracellular environment is also important in main-

taining high Wg levels within the epithelium. Cells overexpressing the Glypican HSPG, Dally-like, accumulate higher than normal levels of extracellular Wg, whereas clones defective in HSPG biosynthesis bind lower levels of extracellular Wg than normal and are deficient in Wg signaling (Baeg et al., 2001).

Why constrain morphogen movement in tissues? Using cell-surface receptors and proteoglycans to locally modify the composition of the extracellular environment, cells can help shape morphogen gradients. In fact, expression levels of morphogen receptors and of proteoglycans are spatially regulated. If ligands travel by diffusion, the dense cell-surface proteoglycan sheath might also facilitate retention of the ligands close to the cell surface, where their receptors are located. Many of the tissues in which morphogen gradients form are epithelial. The *Drosophila* leg imaginal disc illustrates why it is necessary to constrain ligand movement to the plane of the epithelial sheet. Wg and Dpp form long-range gradients that pattern the leg along the proximal-distal axis. They activate distal genes close to the center of the disc and repress proximal genes toward the periphery. Since the leg disc is highly folded, the distal-most tip of the leg is very close to the proximal leg segments and body wall in the disc (Figure 2). If Wg and Dpp were to diffuse out of the plane of the epithelium, distal structures might erroneously be specified as being proximal. The observations of Lewis et al. (2001) are particularly interesting in this context. Perhaps cholesterol modification is needed for efficient retention of Shh. The reduced effectiveness of unmodified Shh in signaling to distant cells might be explained if it is more easily lost.

A Special Case: Transport of Ligand by Cell Movement

As tissues grow, cells are passively displaced away from a morphogen source. Stable interaction of secreted proteins with the extracellular matrix raises the possibility that morphogens could be passively carried along with cells as they move. Pfeiffer et al. (2000) have asked whether cell-mediated ligand transport could contribute to patterning in the *Drosophila* embryo. They replaced the endogenous Wg gene with a membrane-tethered form of Wg that cannot be secreted. They found that tethered Wg rescued many of the patterning functions of Wg in the embryonic ectoderm that have previously been ascribed to action of secreted Wg at a distance. This can be explained by the observation that some of the progeny of Wg expressing cells are displaced anteriorly by cell division in the embryonic segment. These cells can carry tethered Wg with them and pattern the ectoderm to which they contribute. Although Wg is normally secreted in the embryo, this study demonstrates the possibility that cell-based transport can contribute to ligand movement in tissues.

Does passive, cell-mediated transport contribute to long-range gradient formation in the imaginal discs? In the case of the Wg and Dpp gradients, it has been possible to interrupt ligand secretion and allow the endogenous gradients to disperse. Subsequently, ligand secretion was reinitiated and the time course of de novo gradient formation could be assessed. In both cases, new long-range gradients formed very rapidly, indicating that cell movement and growth do not contribute significantly to gradient formation in these tissues (Entchev et al., 2000; Strigini and Cohen, 2000; Teleman and Cohen, 2000). It will be of interest to see whether passive, cell-associated transport is used in other systems.

Can Diffusion Be Ruled in or out?

We are beginning to see evidence in support of the idea that active vesicle-mediated transport can contribute to morphogen movement. There is also evidence that seems more compatible with extracellular gradient formation. We now face the problem of how to critically evaluate the extent to which passive diffusion contributes to gradient formation. Although we favor diffusion as the likely mechanism of Wg movement, diffusion is very difficult to confirm or to definitively rule out. The rate of Activin diffusion has been estimated in the range of 100 microns per hour, in excess of what would be needed to explain Wg or Dpp gradient formation in the wing disc. Current estimates for the rate of vesicle movement observed within cells are considerably faster, however, nothing is known about the effects of endocytosis and resecretion on the rate of net movement. It is possible that more precise measurements of the rates of ligand movement may help to address these issues. Another possibility might be to test whether the rate of gradient formation is temperature dependent. Diffusion is likely to be less temperature dependent than active transport. Experiments to test the passive diffusion model now pose a challenge to everyone interested in gradient formation.

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