Sonic hedgehog: making the gradient

The amino-terminal peptide of Sonic hedgehog is a cell-tethered molecule, which nevertheless seems to provide a developmental signal that acts at a distance and has different effects depending on its concentration. Recent structural data suggest that zinc-dependent proteolysis may somehow be involved in Sonic hedgehog's function.

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Embryonic development is regulated by the coordinate action of secreted factors synthesized by discrete groups of cells. Cells in the vicinity of these signaling centers respond by adopting different fates. In this way, the diverse array of cell types in the embryo is specified. One of the most intriguing discoveries of the past decade is that the molecular identities of many of these factors are conserved across phyla, from *Drosophila* to mouse, despite gross differences in body plans. A case in point is the Hedgehog family of secreted proteins.

The *Hedgehog (Hh)* gene was first identified in *Drosophila* where it has been shown to mediate crucial patterning events at several points during development [1]. Firstly, as a member of the segment polarity class of genes, *Hh* is required for establishing the arrangement of cell types in each segment along the anterior–posterior axis. *Hh* mutants thus exhibit a loss of a particular region of each embryonic segment. Later in development, *Hh* is necessary for patterning the imaginal discs, which are the precursors of the legs, wings, eyes and other structures of the adult fly. In both of these cases, Hh protein seems to act over a very short distance to induce the expression of other signaling proteins in a stripe of neighboring cells.

A third activity of Hh during *Drosophila* development is in patterning of the dorsal epidermis [2]. In each embryonic segment, four different types of epidermal cells can be distinguished by the morphology of cuticular projections which they secrete, and the position that they occupy along the anterior–posterior axis. The proximal–distal arrangement of these cell types, relative to the site of Hh synthesis at the parasegment border, is invariantly repeated in every segment. Using heat-shock-inducible and temperature-sensitive forms of Hh, Heemskirk and DiNardo [2] demonstrated that modulation of the level of Hh signal altered epidermal-cell pattern. Increasing the amount of Hh led to an increase in the number of proximal epidermal cells at the expense of distal cells. Conversely, decreasing the amount of active Hh protein caused proximal epidermal cells to adopt distal cell fates. Hh thus seems to act as a morphogen, with high concentrations specifying proximal and low concentrations specifying distal epidermal cells. This long range requirement for Hh distinguishes it from the short range activities in early segment patterning and imaginal disc development.

A family of genes homologous to Drosophila *Hh* has recently been identified in vertebrates [3]. One family member, *Sonic hedgehog* (*Shh*, also known as *vhh-1* and *Hhg-1*) has been shown to mediate a variety of important signaling functions. Sequence comparisons revealed that the amino-terminal halves of Hh proteins from invertebrates and vertebrates are far more highly conserved than the carboxyl portions. Biochemical studies of Hh proteins synthesized *in vitro* and *in vivo* showed that they are proteolytically cleaved to produce two secreted peptides: a 19-kDa amino-terminal peptide and a 27-kDa carboxy-terminal peptide [4–6]. Further work using proteins expressed in bacteria led to the surprising finding that this cleavage is autocatalytic, and depends on conserved sequences in the carboxyl portion [7]. Moreover, the cleavage occurs within an invariant tripeptide that defines the carboxy-terminal end of the most highly conserved part of Hh proteins [8]. This raised the tantalizing possibility that two distinct signaling molecules might exist, one performing conserved functions and the other having divergent roles. But functional studies with both *Drosophila* and vertebrate Hh proteins appear to rule out this possibility, as it seems that all of the signaling activity resides in the amino-terminal peptide.

The conserved spatio-temporal expression of *Shh* in mouse, chicken and zebrafish embryos immediately suggested that it is a potential mediator of several crucial inductive events in vertebrate development [3]. These include patterning of the central nervous system, somites and limbs. One area of high level Shh expression is the notochord — a rod-like structure running most of the length of the embryonic axis beneath the developing brain and spinal cord. Early studies had demonstrated that the notochord has the ability to induce the development of the floor plate, a specialized group of cells in the ventral-most part of the neural tube [9]. In fact, co-culturing pieces of neural tissue that would not normally form floor plate with notochord explants leads to the induction of floor plate cells [10]. This induction requires contact between the notochord and the responding tissue, suggesting that either a cell-bound factor, or a very high concentration of a diffusible factor is required. The notochord and floor plate share another inductive capacity, the ability to induce motor neurons. Unlike floor plate induction, motor neuron induction is not contact dependent, implying the presence of a diffusible inducing factor [11].

Shh protein is present in the notochord and floor plate at the right time to account for both the floor plate and motor neuron inducing activities of these axial structures [12]. Moreover, recombinant Shh amino-terminal peptide, but not carboxy-terminal peptide, is capable of substituting for these tissues in the induction of floor plate and motor neurons *in vitro* [13,14]. Induction of floor plate requires a higher concentration of Shh than does motor neuron induction [14]. Shh, like *Drosophila* Hh, can thus be thought of as a morphogen inducing different neural cell types in a concentration-dependent manner.

The notochord also influences development of the somites, a series of paired mesodermal blocks adjacent to the neural tube. These initially homogeneous structures differentiate into the ventral–medial sclerotome and dorsal–lateral dermomyotome. The sclerotome gives rise to skeletal elements of the ribs and vertebrae. The dermomyotome further differentiates into the dermotome, which gives rise to the dermis and to the myotome, from which the axial musculature develops. The initial event in somite development, sclerotome induction, is mediated by the notochord, and notochord fragments can induce sclerotome development in explanted presomitic mesoderm [15]. Purified Shh amino-terminal peptide can substitute for the notochord in this induction. Shh also influences myogenesis [15–17]. Thus, Shh synthesized at the axial midline can influence development across the entire width of the somite.

Shh also has a crucial role in limb development. In the limb buds, *Shh* RNA and protein are expressed in a small region of posterior mesoderm that corresponds almost exactly with an experimentally defined region termed the zone of polarizing activity, or ZPA [12,18]. The ZPA is responsible for imposing pattern on the developing limb; it is necessary for specifying anterior–posterior identities of long bones and digits. When ZPA tissue is grafted to the anterior side of a host limb bud, extra digits develop with mirror-image symmetry to the normal ones. The extent of digit duplication depends on the number of ZPA cells transplanted; anterior digit duplication requires fewer cells than does posterior digit duplication [19]. This suggests that the ZPA is the source of a secreted factor that forms a gradient across the limb bud and specifies each digit at a distinct concentration threshold. The amino-terminal peptide of Shh can substitute for the ZPA in causing digit duplication [20]. As for ZPA grafts, the extent of digit duplication depends on the amount of Shh protein applied (C. Tickle, personal communication). Thus Shh has the ability to influence patterning across the entire limb bud.

It is clear from these studies of *Drosophila* and vertebrate Hh activity that the proteins exert both long and short range influences on a variety of tissues. One could easily envision a model wherein Hh synthesized from a group of cells diffuses and forms a concentration gradient across the *Drosophila* epidermis and imaginal disc, the vertebrate neural tube, somite and limb bud. High Hh concentration near the site of synthesis would mediate short range signaling, while the low concentrations found at a distance would be sufficient for long range activities. Unfortunately, this simple diffusion model does not jibe with the reality of Hh biochemistry.

When full-length Hh proteins are expressed in tissue-culture cells, autocatalytic processing generates the amino- and carboxy-terminal peptides. The amino-terminal peptide does not diffuse significantly from the cells, however, and is barely detectable in the culture medium ([7] and D.A.B., unpublished results). Instead it remains tethered at the cell surface. The mechanism of this cell attachment remains mysterious, but it presumably involves

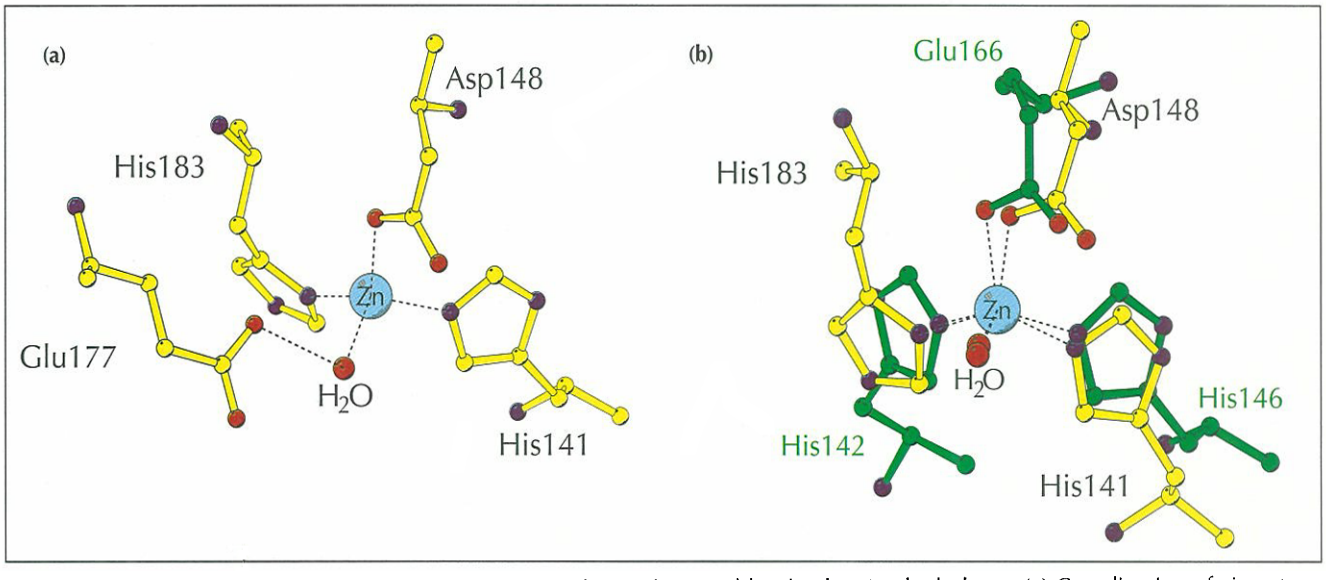

Fig. 1. The coordination of zinc by the Shh amino-terminal peptide resembles that by zinc hydrolases. **(a)** Coordination of zinc atom (shown in turquoise) by Shh. Amino acid side chains are shown in yellow with red oxygen atoms and blue nitrogen atoms. The zinc-bound water molecule is shown as a red oxygen atom. The proposed catalytic glutamate (Glu177) is also shown. **(b)** Superposition of zinc coordination by Shh (yellow side chains) and thermolysin (green side chains). Reprinted with permission from [22].

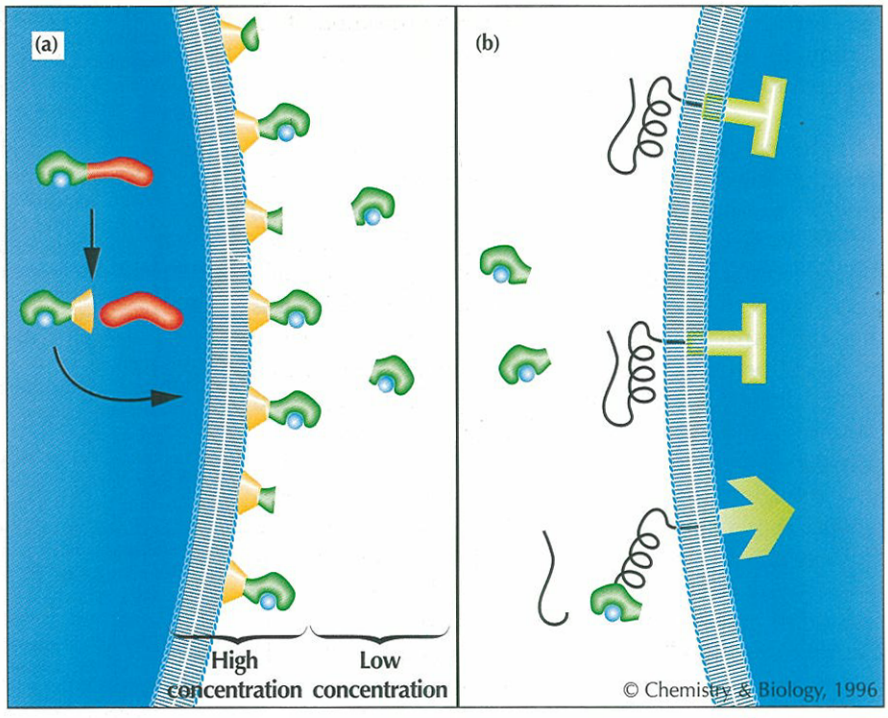

Fig. 2. Possible roles for the proteolytic activity of the Shh amino-terminal peptide. **(a)** The Shh amino-terminal peptide (green) is shown tethered to the cell surface via a modification at its carboxyl terminus (yellow). This modification occurs during autocleavage, which is dependent on sequences in the carboxyl portion. Some of the cell-bound Shh may then be released for long range signaling by the zinc-dependent proteolytic activity of Shh. The coordinated zinc atom is shown in turquoise. **(b)** Shh amino-terminal peptide may activate its receptor by proteolytic processing. The arrow indicates signaling by an activated receptor.

a modification at the end of the amino-terminal peptide that occurs during autocleavage; a truncated form of Shh, which terminates precisely at the cleavage site, is not modified in this way, and, indeed, diffuses freely from the cell ([8] and D.A.B., unpublished results).

How, then, can a cell-attached factor mediate long range signaling events? One possibility is an indirect mechanism, in which the local signal induces the expression of a second, diffusible factor that has long range activity. Such a mechanism is probably at work in the *Drosophila* imaginal disc where transcription of the *decapentaplegic* (*dpp*) gene, which encodes a secreted protein of the TGF-β superfamily, is activated by Hh. Dpp then mediates long range signaling activities [21]. In other instances of long range Hh signaling, epidermal patterning in *Drosophila* and motor neuron induction and somite patterning in vertebrates, secondary signals have not been identified, suggesting a direct role for Shh. If this is so, Shh needs to be liberated from the cell surface to diffuse over several cell diameters. A model that explains how this may occur has been proposed from studies of the Shh crystal structure [22]. In these studies, a bacterially expressed protein containing most of the mouse Shh amino-terminal peptide was crystallized, and the structure was solved at 1.7 Å resolution. The protein fold seen in the structure, which contains two α helices and multiple β sheets connected by extensive loops, has not been observed previously. Surprisingly, electron-density analysis and atomic-absorption spectroscopy revealed a zinc atom that is coordinated by three amino acids and a water molecule. The tetrahedral coordination of zinc by Shh is similar to that found in zinc hydrolases such as thermolysin and carboxypeptidase A (Fig. 1). This differs from structural zinc coordination like that found in 'zinc finger' transcription factors, in which four amino-acid residues coordinate the metal and sequester it in a hydrophobic pocket. In Shh and zinc hydrolases, a water molecule participates in the zinc coordination, and the metal is within a solvent-exposed region of the protein. This is consistent with the idea that the zinc atom has a catalytic role. The presence of a glutamate residue in Shh in the vicinity of the coordinated zinc atom, a position analogous to that of the glutamate required for catalysis by zinc hydrolases, further supports the hypothesis that Shh may have catalytic activity.

If Shh has proteolytic activity, what is the substrate? Two possibilities are suggested (Fig. 2). First, the activity could be directed against cell-tethered Shh itself, thus releasing the molecule for long range activity. The arrangement of Shh molecules in the crystal lattice supports the notion of self-directed activity. It was observed that the carboxyl terminus of one molecule was inserted into a crevice in a second molecule very near the zinc coordination site. This arrangement suggests that the role of Shh proteolytic activity may be to hydrolyze a peptide bond near the carboxyl terminus of a neighboring Shh molecule. Perhaps, when Shh synthesis reaches a critical level, the molecules are sufficiently concentrated within the secretory pathway or on the cell surface to favor proteolytic release of some of the protein. This could produce very steep concentration gradients of Shh within the embryo.

Alternatively, Shh may proteolyze a different substrate, thus leaving open the question of how Shh is released from the cell surface (though, with time, it may be released by general extracellular proteases). One substrate could be an Shh receptor. It is possible that the Shh receptor must be proteolytically processed by the ligand to effect signaling. An example of such a mechanism is

the cleavage of the thrombin receptor by thrombin. This is thought to unmask a domain on the receptor that is required for activation [23].

The two models outlined above are attractive, but it must be emphasized that, as yet, the Shh amino-terminal peptide has not been proven to have any proteolytic activity. In fact, *in vitro* assays, using a peptide encompassing a potential cleavage site near the end of Shh as a substrate, failed to detect cleavage. A possible explanation is that Shh is inactive as a protease or a substrate unless it is modified, perhaps by the carboxy-terminal addition that occurs during autocleavage. Thus, bacterially expressed protein would not be active. Another surprising fact is that the *Drosophila* Hh protein lacks two of the amino acids thought to be crucial for zinc coordination and proteolytic activity. Does this mean that Hh signaling in the fly does not depend on this activity? Could the proteolytic activity required either to release the protein or to activate its receptor be provided by an additional factor? Clearly, some solid biochemistry needs to be done to fully understand the capabilities of this fascinating molecule in regulating the invertebrate and vertebrate body plans.

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