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Hedgehog Processing and Biological Activity

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Introduction

One of the fundamental questions in development is how cells acquire their individual identities within an embryo to form the proper body pattern. During embryogenesis, each cell is specifically situated within a morphogenic field, where it develops a particular function. This function is determined primarily by the various extracellular signals received by these cells. One example of such an extracellular morphogenic signal is the Hedgehog (Hh) family of secreted proteins, which have been shown to specify numerous cell fates in a concentration dependent manner. Besides its role as a morphogen, Hh family members have also been shown to act as a morphogen, mitogen, cell survival factor and axon guidance factor. Consistent with the important role Hh family members play in both human embryonic development, and in tissue regeneration within the adult, inappropriate Hh signaling results in numerous pathological conditions, such as holoprosencephaly, Gorlin's Syndrome and cancer. Given the importance of Hh signaling to human development and disease, numerous laboratories have been working to elucidate the unprecedented biology underlying Hh signaling, including the use of numerous invertebrate and vertebrate model systems. This review is intended to give a generalized overview of the biosynthesis and signaling of the Hh family of proteins and throughout the rest of this text will use the term Hh to generalize all the described family members, regardless of the model system in which the work originated. For more detail, several more comprehensive reviews exist about specific components and differences between invertebrate and vertebrate pathways [46-49].

Hh was originally discovered in a classic genetic screen performed by Nusslein-Volhard and Wieschaus, from which they identified mutations that disrupt the cuticle patterning of the *Drosophila* embryo [1]. Mutation of *Hh* gives the embryo a dramatic 'spiny' phenotype reminiscent of those seen on the ubiquitous old-world mammal, hedgehog. Three vertebrate *Hh* orthologs were subsequently identified, *Sonic hedgehog* (*Shh*), *Indian hedgehog* (*Ihh*) and *Desert hedgehog* (*Dhh*). Though somewhat redundant, *Ihh* and *Dhh* expression appear to be more tissue specific, while the expression of *Shh* appears most widespread [4-6]. *Shh* null animals have a multitude of defects, including cyclopia, poor neural patterning, lack of limb growth and abnormal organ and foregut development [4]. *Ihh* null mice are most noticeably deficient in aspects of bone development, whereas *Dhh* null mice exhibit defects in spermatogenesis and in nerve sheath development. Mice deficient for both *Ihh* and *Shh* die at approximately E10.5, much earlier than mice lacking either gene product on its own, consistent

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with the various family members being redundant for certain functions. In general, the processing and signaling of Hh family members is evolutionarily conserved, although some animal specific signal modulators have also been described.

Production and Lipid modification of Hh

Hh is unique in that it is the only known secreted ligand that is covalently modified by cholesterol, probably resulting in many of the unprecedented biological mechanisms that have been described in the Hh pathway. Hh is initially synthesized as pre-protein that enters the secretory pathway, where the signal sequence is removed to yield an approximately 45 kDa precursor molecule. This full-length protein is subsequently cleaved into two discrete peptides at a highly conserved three amino-acid motif, GlyCysPhe, to yield an amino-terminal domain (HhN) and a carboxy-terminal domain (HhC). This internal proteolytic event results in the concomitant attachment of a cholesterol moiety to a Gly residue in the newly formed carboxy-terminus of HhN, resulting in HhNp (where “p” stands for processed) [7,8]. This cleavage is thought to occur in an intramolecular manner, with the HhC domain acting as a cholesterol transferase. Both the internal cleavage and covalent modification by cholesterol occur as result of two specific nucleophilic displacement reactions [8]. First, the sulfur that resides within the side chain of the conserved Cys attacks the carbonyl of the adjacent Gly causing an internal molecular rearrangement. This shift allows for a second nucleophilic attack to occur on the same carbonyl by the cholesterol molecule's hydroxyl group, resulting in the cleavage of the bond between the carbonyl and sulfur. The addition of cholesterol to the amino-terminal peptide is the result of the thiol displacement, which remains with the carboxy-terminal peptide to form an ESTER LINKAGE.

The mature form of Hh is also modified by the addition of a second lipid molecule, palmitate. This lipid modification to Hh is thought to be directly regulated by a membrane-bound O-acyltransferase (MBOAT) commonly known as Skinny Hedgehog (Ski), which adds a palmitate to the amino-terminal cysteine exposed by the cleavage of the signal sequence [9, 10]. Unlike the majority of intracellular palmitoylated proteins, the modification of HhN is through an amide linkage and not through the more common thioester linkage. This amide linkage probably gives more stability to this palmitoylation, as palmitoylation through a thioester linkage typically turns over much faster than the protein that is modified. The active site of Ski contains a evolutionarily conserved histidine residue found within all the entire MBOAT family, which when mutated in Ski results in an inactive form of the protein [9,11]. Animals lacking *Ski* exhibit phenotypes reminiscent of *Hh* loss of function mutations, highlighting the importance of palmitoylation for propagation of the Hh signal [9,12].

Lipid modification also serves an important role to promote specificity of its localization and influences Hh movement. Cholesterol is essential for proper trafficking and restricts Hh movement, as it causes insertion into the lipid bilayer and preferential enrichment in membrane lipid rafts [23]. The movement of Hh unmodified by cholesterol is unrestricted, diffusing over longer distances, whereas HhN-p containing the cholesterol moiety is more constrained in its movement. Cholesterol modified Hh is therefore concentrated and can elicit high level responses, whereas unmodified Hh produces a more diffuse low level response, as was predicted by a recent mathematical model of Hh spreading by Saha and Schaffer [24]. Non-lipid modified Hh also interacts less efficiently with HSPG associated protein Shifted (Shf) and HSPGs themselves, diminishing its ability to activate the pathway [25-27]. Guerrero and colleagues found that non-lipid modified HhN diffuses much further than lipidated forms of Hh [28]. Additionally, they found that non-lipid modified HhN has a lower affinity for the plasma membrane, does not interact with HSPGs, and can only activate low level targets [28].

Hh Movement

Hh signaling has been well-characterized in various developmental processes, from the patterning and closure of the neural tube to the specification of asymmetrical digits from the embryonic limb bud. Each tissue has its own characteristic patterning mechanisms, but the fundamental principles remain similar. In general, Hh is produced and secreted from a specialized group of cells contained within a specific compartment of a tissue. Hh moves away from the site of its production and across the developing tissue, where it is received by Hh responsive cells, in effect setting up a morphogen gradient. The cells position in this Hh gradient differentially specifies cell fate, depending on how close the cells are to the source of Hh. Hh can signal over short distances to adjacent cells and up to 200 μm across the developing limb bud. Hh binding to its cell surface receptor, Patched (Ptc), which initiates a cascade of events that results in activation of a distinct set of target genes. At the boundary of the Hh producing and Hh receiving compartments, where the concentration of Hh is highest, the signal is strongest, causing induction of 'high-level' target genes. Further from the Hh source, lower levels of Hh cause the expression of a different set of target genes. The signaling activity of Hh is determined by various factors, such as processing and covalent modification of the ligand, before its release from the intracellular compartment either in a monomeric or a multimeric form. Additionally, its movement across a field of cells is both positively and negatively modulated by a number of cell surface and extracellular players.

The mechanism by which Hh moves through the extracellular matrix has long been a topic of speculation and controversy. The idea that a lipid modified molecule could diffuse freely through the hydrophilic environment of the extracellular matrix seemed paradoxical, as lipid modifications usually serve as membrane anchors. Many models of how Hh might move have been proposed and have included theories of transport via large lipoprotein particles, planar transcytosis, delivery to receiving cells by cytonemes, and active transport. More recent findings presented another model by demonstrating that lipid-modified Hh can form soluble, multimeric molecules, termed s-HhNp [19,20]. Multimeric Hh is found to be secreted into the media of Hh-producing cells. A structural analysis showed that its solubility may be the result of intermolecular interactions that serve to bury the hydrophobic palmitate in a highly conserved pocket. Both protein-protein interactions and protein-lipid interactions are necessary for the formation of the multimeric form of Hh, as Hh that lacks palmitoylation and certain point mutants of Hh cannot form multimers. This multimeric complex which has an apparent molecular weight of ~ 120 kDa, allows Hh to become more freely diffusible, thus able to propagate a long-range signal and is thought to be the most biologically active form of Hh.

Long range signaling has been investigated by gauging movement, diffusion and target gene activation. Various groups have identified different types of large molecular weight particles containing Hh, whose formation was necessary for movement and diffusion through a field of cells. These different particles presumably represent a multimeric form of Hh. Eaton and colleagues demonstrated that lipid-modified proteins, such as Hh and Wingless (Wg), are transported over long distances in lipoprotein particles [21]. They showed that both Hh and Wg copurify and co-localize with lipoprotein particles. Additionally, when lipoprotein levels are reduced, Hedgehog accumulates at the site of production and fails to signal over its normal range, indicating that these lipoprotein particles likely aid the movement of lipid-modified morphogens through the extracellular matrix. Similarly, it was found that cholesterol modification of Hh is necessary for proper trafficking, movement and controlled long-range activity, as the cholesterol moiety causes Hh to assemble into large punctate structures that are differentially targeted to proper subcellular locations via interactions with Disp and HSPGs [18,22].

Release of Hh and Dispatched

In order for fully processed HhNp to signal in a long range fashion, it must first be released from the cells that are responsible for producing it. The putative twelve-pass membrane protein Dispatched (Disp) has been implicated in the release of cholesterol-modified HhNp [14]. Disp contains a sterol-sensing domain and shares homology with prokaryotic Resistance-Nodulation-cell Division (RND) permeases [14,15]. Hh processing occurs normally in *disp*^{-/-} cells, but it is unable to leave the cell [14]. *Disp*^{-/-} cells are, however, able to release HhN, implying that Disp can only interact with the cholesterol modified form of Hh, HhNp [14]. Complete loss of *disp* function leads to strong Hh loss of function phenotypes, indicating a positive role for Disp in Hh signaling [14]. Furthermore, conditional knockout of *Disp1* in only the *Shh* producing cells produces the same phenotype, indicated that *Disp* is only required in cells that produce Shh [16,17]. Expression of ShhN can only partially rescue this phenotype, consistent with the requirement for the cholesterol modified form in long range signaling [17]. Another role has been suggested for Disp in the sorting of Hh within polarized epithelial cells [18]. Hh localizes to two distinct regions of the cell, within the polarized epithelial cells where it is made, near basal or apical membranes [18]. These sites of localization are consistent with two distinct routes of exit for HhNp, as differential effects on these two pools of HhNp are observed in various genetic backgrounds. Dispatched (Disp) plays a pivotal role in the release of at least one of these HhNp pools, as HhNp produced from cells lacking Disp lose the ability to signal over long distances, but can still affect cells closest to the HhNp producing cells [18].

Hh Activity

It has been shown that there are two main forms in which Hh exists; monomeric and multimeric. The monomeric form of Hh is most often associated with short-range signaling and has less activity relative to multimeric Hh. The multimeric form of Hh is responsible for long-range signaling and has the greatest activity, which appears to be associated with its ability to freely diffuse [19,20]. A structure-function analysis of Shh mutants demonstrated a link between activity and their individual ability to form multimers [19]. Furthermore, ShhN mutants that lack the palmitoylation site still bind to Ptc with affinity comparable to wtShh [29], providing further evidence that higher activity of Shh is due to palmitoylation, and thus multimerization, as the addition of palmitate is necessary for multimer formation [19].

Lipid modification is an important aspect when considering level of Hh activity and strength of signal. The link between modification of Hh and activity becomes apparent when one compares the activity of recombinant Hh, which is not lipid modified, to that of endogenous Hh. Presumably, the only difference between the two is the lack of lipid modifications on the recombinant form, but recombinant Hh has much less potency than lipid modified Hh. Hh that has been dually modified by both cholesterol and palmitate is considered to be the most active form, producing highest level pathway activation. Although cholesterol modification does not appear to be essential for activity, it has been suggested that its main role is to ensure the proper spreading of the signal and to prevent signal dilution.

Control of Movement by Extracellular Proteins

Control of morphogen movement is an important aspect of regulating their effects, as movement and activity tend to be closely linked. Hh must be able to move to its target tissues, but in a precise and controlled manner. This is accomplished by extracellular regulatory proteins that serve as both negative and positive pathway regulators by controlling movement of Hh. Several of these accessory factors have been identified in various animals, but do not appear to be evolutionarily conserved. However, the need for mechanisms to regulate Hh movement is conserved.

The endocytic receptor, megalin, is a member of the LDL family of receptors that has been implicated in Hh signaling [30,31]. Hh binds to megalin with high affinity and megalin mediates Hh internalization through an unknown mechanism. Mice that lack megalin exhibit defects in forebrain development and a holoprosencephalic phenotype reminiscent of Shh knockout mice [32], indicating that megalin acts primarily as a positive regulator of the pathway, perhaps by enhancing Hh binding to its receptor Patched (Ptc). Ptc is a twelve-pass transmembrane protein that contains a sterol-sensing domain and plays its own role in sequestering Hh as well [33]. Hh binds to Ptc, activating the pathway and thus upregulating target genes, one of which is *Ptc* itself, serving to increase the concentration of Ptc where there is highest level pathway activation. The presence of more Ptc binds up free Hh, limiting the range of its spread. Like Ptc, Hedgehog-interacting protein (Hip) is also upregulated by Hh signaling and binds to Hh to inhibit its movement [33]. Hip is a primarily membrane bound extracellular protein and when its expression is induced by Hh signaling, it creates a self-limiting feedback mechanism within the pathway [33-35]. Another protein, the Wnt-inducible growth-arrest specific gene (*Gas1*) was also thought to attenuate signaling by antagonizing the diffusion of Hh by binding to it [36]. However, more recent evidence also shows a positive role for *Gas1* in Hh signaling. Embryos that lack *Gas1* have a phenotype similar to that of *Shh* null embryos, including neural tube and craniofacial defects [37,38]. *Gas1* enhances signaling by promoting Hh binding to Ptc and cooperation with CDO, another cell surface protein that positively modulates the Hh signal [37,38]. CDO belongs to a group of proteins termed the interference hedgehog family, which includes BOC and interference hedgehog (Ihog) itself. These three proteins mediate signaling by their interactions with the Hh ligand via fibronectin type III domains and are thought to enhance Hh binding to Ptc in vivo [39, 40].

Role of Heparan Sulfate Proteoglycans

Heparan sulfate proteoglycans (HSPGs) are very large extracellular molecules that consist of heparan sulfate glycosaminoglycan polymers covalently linked to a protein core, which have been implicated in the regulation of Hh signaling and formation of the morphogen gradient. They function as regulators of many ligands at the cell surface and have been found to aid in the formation of receptor complexes. The HSPGs that have been associated with Hh signaling usually have protein cores composed of either syndecan-3 or glypicans. These macromolecules are attached to cell surface membranes, usually by a transmembrane domain or a GPI-anchor. Hh interacts with HSPGs through a highly conserved Cardin-Weintraub sequence found in its N-terminus, creating an electrostatic interaction between the negatively charged sulfates of the HSPGs and Hh [41]. HSPGs have been found to interact with other extracellular matrix factors that mediate interactions with Hh, such as Shf, as well as lipid-modified Hh carried in large punctate structures [26,27].

The first evidence of HSPG involvement in the Hh pathway came from embryos mutant for tout-velu (*ttv*), which encodes a glycosyltransferase required for heparan sulfate polymerization and is homologous to the mammalian tumor suppressor family of exostosin (EXT) glycosyltransferases. These mutant animals exhibited a Hh-like loss of function phenotype when mutated, such that Hh is unable to move properly in the absence of these glycosyltransferases and fails to activate target genes, demonstrating a role for HSPGs in the facilitation of Hh movement [25,42].

One stage specific HSPG regulator is the secreted protein sulfatase 1 (*Sulf1*), which is found in the ventral portion of the neural tube where it acts as a positive mediator of Hh signaling [43]. *Sulf1* temporally regulates the sulfation state of HSPGs to create Shh binding sites, thus concentrating the amount of Shh delivered to the receiving specific neural progenitor cells, effectively switching their cell fate from a neuronal lineage to that of oligodendrocyte

precursors. Additionally, two genes that encode glypicans, division-abnormally-delayed (dally) and dally-like (dlp), were investigated for a role in the Hh pathway. Through genetic studies and RNA interference, only Dally-like was shown to be necessary for Hh signaling, as loss of Dally-like prevents extracellular Hh distribution [44,45]. This loss-of-function phenotype seen with *dlp* indicates an overall positive role for HSPGs in Hh signaling.

Concluding Remarks

Regulation of Hh activity is determined by a multifaceted system that involves an unusual processing mechanism, lipid modification, and a host of extracellular regulators of movement. Hh's ability to signal over a range of distances and affect cell fate is both a function of its degree of processing and modification, but also the accessory factors that control its diffusion. It is clear that Hh regulation is important, as demonstrated by its many roles as morphogen, mitogen, cell survival factor in both development and various cancers.

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Name	Form of Hh	Activity	Physiological Relevance
Unmodified		+	None
Palmitoylated		+++	None
Cholesterol modified		++	Observed <i>in vivo</i>
Modified by palmitate & cholesterol (monomer)		++++	Observed <i>in vivo</i>
Modified by palmitate & cholesterol (multimer)		++++	Observed <i>in vivo</i>

Figure 1. Hh activity

Hh can exist in multiple forms, each with varying levels of activity and physiological relevance. Hh that is dually modified has been shown to have more activity than unmodified Hh, but overall palmitoylation appears to have the most effect on activity. Unmodified Hh and Hh that has been singly modified by palmitate have not been found to exist *in vivo*.

Figure 2. Overview of Hh production and movement

The Hh producing cell shown in the left panel outlines the various steps in Hh modification before its release by Disp. Hh is produced as a pre-protein, represented in three domains; a signal sequence (S-yellow), an amino-terminal domain (N-blue), and a carboxy-terminal domain (C-red). The signal sequence is cleaved to produce a full-length unmodified form of Hh. An autocatalytic reaction removes the carboxy-terminus and attaches a cholesterol moiety (C-green) to the newly formed carboxy-terminus. Hh is then further modified by the addition of a palmitate (P-orange), a reaction catalyzed by Ski. The dually modified form of Hh is then released by Disp to the cell surface as a monomer or to the extracellular compartment in a multimeric form. The right panel represents a Hh receiving cell. The movement of Hh is aided or inhibited by various factors. Proteins such as HSPGs, megalin and the family of Ihog proteins serve as positive regulators (green), either by promoting its movement, enhancing the Hh signal or assisting its binding to Ptc. Negative regulators (red), such as Hip and Ptc, prevent Hh movement or serve to bind up excess Hh. Freely diffusible factors have been identified in some animals and are represented as binding to Hh in the extracellular matrix. Ultimately, Hh must bind to its receptor, Ptc, in order to activate the transcription of various target genes.