Smoothened Goes Molecular: New Pieces in the Hedgehog Signaling Puzzle*

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A general aim of studies of signal transduction is to identify mediators of specific signals, order them into pathways, and understand the nature of interactions between individual components and how these interactions alter pathway behavior. Despite years of intensive study and its central importance to animal development and human health, our understanding of the Hedgehog (Hh) signaling pathway remains riddled with gaps, question marks, assumptions, and poorly understood connections. In particular, understanding how interactions between Hh and Patched (Ptc), a 12-pass integral membrane protein, lead to modulation of the function of Smoothened (Smo), a 7-pass integral membrane protein, has defied standard biochemical characterization. Recent structural and biochemical characterizations of Smoothened domains have begun to unlock this riddle, however, and lay the groundwork for improved cancer therapies.

Members of the Hedgehog (Hh) family of secreted signaling proteins are present in most metazoans and owe their name to the effects that loss of Hh function has on Drosophila embryos, which lose their normal segmented pattern and develop a uniform coat of bristles reminiscent of the coats of hedgehogs (1). As presaged by this phenotype, Hh proteins mediate essential tissue patterning events during many stages of animal development (2), and abnormal Hh function is associated with birth defects and cancer (3). Hh proteins are also involved in tissue maintenance and wound repair in adult animals (4). Hh proteins achieve their patterning effects by functioning as classical morphogens (5). That is, Hh proteins form gradients of decreasing concentration from sites of secretion and induce concentration-dependent differentiation of distinct cell types (6, 7). As befits a morphogen, Hh expression, release, diffusion, and signal reception are tightly regulated by multiple factors (8).

Classical and modern genetic techniques have identified several cell-surface proteins and glycans involved in receiving or modifying Hh signals (9). The core components of this process, conserved in all organisms known to have active Hh signaling, are Patched (Ptc) and Smoothened (Smo) (Fig. 1) (10–13). Ptc functions upstream of Smo and has been genetically and biochemically defined as a primary component of the Hh receptor (14, 15). Ptc is a 12-pass integral membrane protein with distant homology to bacterial resistance-nodulation-cell division (RND) transporters (16, 17). Transmembrane helices 2–6 of Ptc are also homologous to sterol-sensing domains, which are found in diverse integral membrane proteins and regulate activity in response to levels of free cellular sterols (18). Smo is a member of the Frizzled family (class F) of G-protein coupled receptors (GPCRs) (19), and contains an N-terminal, ~14-kDa extracellular cysteine-rich domain (CRD) connected via a linker to 7 membrane-spanning helices (7TM) and an extended (~200 amino acids, human; ~450 amino acids, Drosophila) C-terminal tail.

Hh signaling responses are modulated by additional cell-surface components including CDO, BOC, Gas1, Hedgehog-interacting protein, and glypicans in vertebrates and Ihog, BOI, and the glypican Dally-like protein in flies (20–29). These factors either lack intracellular regions because of glycophaspatidylinositol anchors (Gas1, glypicans) or have intracellular regions that are not implicated in Hh signaling and do not appear to transmit Hh signals across the cell membrane directly (14). Instead, transmission of Hh signals across the membrane appears to be mediated by Smo, the most downstream cell-surface component of the Hh signaling pathway. Consistent with this role, the cytoplasmic tail of Smo becomes heavily phosphorylated and likely changes disposition when the Hh pathway is active (30–32). These changes are coupled to intracellular signaling events that ultimately converge on members of the Gli family of transcription factors, active forms of which translocate to the nucleus and up-regulate expression of target genes (33).

Recent discovery of the importance of Ptc and Smo localization for normal Hh signaling has added additional complexity to Hh pathway regulation. In vertebrates, Sonic Hh (Shh) and Hh pathway agonists result in movement of Smo from the plasma membrane to the primary cilium, a nonmotile flagellar-like organelle present on most cells, and dispersal of Ptc from its previous localization at the base of the primary cilium (34). Although movement of Smo to the primary cilium appears essential for normal Hh signaling in vertebrates (35), this movement is neither sufficient for signaling (36) nor conserved in flies (37), and a core signaling capacity that is independent of ciliary localization must be present in Smo. This minireview will focus on recent advances in structural and biochemical characterization of Smo, and readers are encouraged to consult other sources for background on additional Hh pathway components.

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3 The abbreviations used are: Hh, Hedgehog; Ptc, Patched; Smo, Smoothened; GPCR, G-protein coupled receptor; CRD, cysteine-rich domain; 7TM, 7-transmembrane; Shh, sonic hedgehog; βAR, β-adrenergic receptor; ECL, extracellular loop; ANTA XV, 2-(6-[(4-benzyl)phthalazin-1-y]piperazin-1-y]pyridin-3-yl)propan-2-ol; 20(S)-OHC, 20(S)-hydroxycholesterol; 22(R)-yne, alkyne derivative of 22(R)-hydroxycholesterol; 20-keto-yne, derivative of 20(S)-OHC with the hydroxyl group converted to a ketone; CCM, cholesterol consensus motif; BCC, basal cell carcinoma.
Patched and Smoothened

In unstimulated Hh-responsive cells, Ptc functions upstream of Smo to inhibit its activity (2). Hh triggers signaling responses by interacting with Ptc to relieve this inhibition, but both how Ptc inhibits Smo and how Hh relieves this inhibition remain unclear. As a small amount of Ptc is sufficient to inhibit a large stoichiometric excess of Smo (16), Ptc does not appear to inhibit Smo through a direct interaction. Rather, the homology of Ptc to transporters and the ability of Smo activity to be modulated by small molecules have led to the widespread belief that Ptc controls Smo through transport of a small molecule intermediary (16). Indeed, the ability of Smo to bind and be inhibited by the plant sterol cyclopamine led to the development of compounds targeting the cyclopamine-binding site for the treatment of cancers with abnormally active Hh signaling (38, 39). As some Smo-binding compounds function as Hh pathway agonists, it has been tempting to speculate that an endogenous cyclopamine-like compound modulates Smo activity (40).

Smoothened: 7TM Region

The absence of knowledge of the physiological factors responsible for Smo activation (or inhibition) has presented a frustrating barrier to understanding Hh pathway regulation, but several recent results have begun to clarify this issue. Firstly, Stevens and colleagues (42–44) have determined atomic resolution crystal structures of the 7TM region of human Smo complexed with five different small molecules, including cyclopamine. As some Smo-binding compounds function as Hh pathway agonists, it has been tempting to speculate that an endogenous cyclopamine-like compound modulates Smo activity (40). Indeed, the sterol vitamin D3 has been proposed to function as a Ptc-dependent inhibitor of Smo (41), although this observation awaits confirmation.

FIGURE 1. Major transmembrane components of Hh signal reception and transduction. Ptc (left) represses Smo (right) through an unknown, indirect mechanism. The interaction of Sonic hedgehog N-terminal domain (ShhN) with Ptc relieves Ptc-mediated repression of Smo. The sterol-sensing domain of Ptc (TM II–TM VI) is colored blue. For Smo, the 8 cysteines mediating 4 disulfide bonds in the Smo ECLs are shown in green; D473H, a Vismodegib resistance mutation, is in blue; W535L, a constitutively activating mutation, is in red; and C-tail sites of serine and threonine phosphorylation (indicated by pS/pT) are in orange.

MINIREVIEW: Smoothened Structure and Function
The five compounds crystallized in the Smo-binding pocket include an agonist (SAG1.5) and four antagonists (LY2940680, SANT1, ANTA XV, and cyclopamine) (see Fig. 4A). All ligands bind in the pocket with their long axes perpendicular to the plane of the membrane but vary in their depth relative to the extracellular outlet (Fig. 2B). At the extremes, cyclopamine interacts predominantly with the extracellular loops, whereas another antagonist, SANT-1, binds deep within the pocket, which spans 28 Å from the top of cyclopamine to the bottom of SANT1. Asp-473, a residue that when mutated to histidine confers resistance to the anti-cancer agent Vismodegib (GDC-0449) (55, 56), lines the drug-binding pocket but interacts differently with different antagonists and does not confer universal drug resistance (43). Asp-473 does not directly contact LY2940680, for example, and the D473H substitution does not affect the activity of LY2940680 (57). The variable susceptibility of individual drugs to resistance mutations suggests that second generation drugs or combination therapies may prolong the time to development of resistance.

LY2940680, cyclopamine, ANTA XV, and the agonist SAG1.5 contact the Smoothened extracellular loops lining the top of the ligand-binding cavity, but SANT1 binds more deeply in the pocket and only contacts ECL2, which is positioned within the 7TM region. In contrast to cyclopamine, which binds more tightly to Smo than to a constitutively active Smo variant bearing a single-site substitution (SmoM2), SANT1 binds both Smo and SmoM2 with equal potency (40). How the position of SANT1 deep within the 7TM bundle correlates to its ability to inhibit both Smo and SmoM2, whose W535L substitution occurs at the base of TM VII, is not clear. Also of interest are the variable effects Smo antagonists have on Smo localization. SANT1, LY2940680, and cyclopamine all inhibit Smo function, but only cyclopamine promotes the translocation of a still inactive Smo to the primary cilium, indicating that translocation and activation are separable functions.

The failure of Smo to adopt an active-like conformation when bound to the agonist SAG1.5 is curious but not unprecedented for agonist-bound GPCRs (58). Binding of an agonist to an apparently inactive state may reflect a low energetic barrier between active and inactive states, conformational flexibility of the active state (59), and/or the effects of truncation of Smo N- and C-terminal regions. SAG1.5 binds in the same region of the binding pocket as LY2940680, ANTA XV, and cyclopamine, and Smo with SAG1.5 bound displays only slight alterations in binding pocket residues. Larger conformational changes associated with active state GPCRs, such as the movements of TMs VI and VII to accommodate G-protein binding, are not seen in the Smo-SAG1.5 structure. Crystallization of an active state of Smo may require adding back the CRD or portions of the C-terminal tail or co-crystallization with active conformation-specific nanobodies (60). Interesting features of the effects of these different drugs on the conformational equilibria of intact Smo and their relation to Smo function clearly remain to be worked out.

Smootherned: Cysteine-rich Domain

A second major insight into Smo regulation emerged when three groups independently showed that oxysterols, oxidized derivatives of cholesterol, bind specifically to the Smo CRD and activate the Hh signaling pathway (61–63). Oxysterol binding by the Smo CRD is functionally as well as physically separable from small molecule binding to the 7TM site as deletion of the Smo CRD results in loss of oxysterol activation of Smo but does not affect the function of agonists and antagonists that target
the 7TM region (61). It had previously been shown that oxysterols could modulate Hh signaling by affecting Smo function (64–66). The site of oxysterol action was not characterized at that time, although oxysterols did not appear to compete with cyclopamine for binding to Smo (66).

The new studies all show that 20(S)-hydroxycholesterol (20(S)-OHC) (see Fig. 4B) activates Smo by binding to the CRD. Additionally, the Rohatgi and Siebold groups (63) were able to determine the crystal structure of the zebrafish Smoothened CRD with residues implicated in binding 20(S)-OHC shown in red (PDB: 4C79).

variable specificity for 20(S)-OHC among Smo CRDs is perhaps not surprising given that the absence of a cellular sterol hydroxylase known to produce it makes it unlikely to be an endogenous ligand (61). Assuming that endogenous ligands for Smo CRDs exist, the question naturally arises of what that ligand is. A survey of oxysterols for Smo modulatory activity found that 7-keto-27-OHC and 7-keto-25-OHC, both metabolites of 7-ketocholesterol, are able to stimulate Hh signaling in a manner that depends on the presence of the CRD (61). Compounds that bind the CRD and inhibit (azasterols, e.g. 22-aza-cholesterol) (Fig. 4B) or partially agonize (20(R))-yne, 20-keto-
residues, Trp-3654.50 and His-3614.46, act as an alternative cholesterol-binding motif presents an intriguing possibility.

Targeting Smoothened in the Clinic

Hh pathway-activating mutations in the gene encoding Ptc, and less commonly the gene encoding Smo, are found in subsets of several cancers, most notably basal cell carcinoma (BCC) and pediatric medulloblastomas (46, 75). Constitutively active mutants of Smo found in sporadic BCC (W535L7.55 “SmoM2”) and more recently in meningiomas and ameloblastomas (W535L7.55, L412F5.51) are resistant to Vismodegib treatment (46, 76–78). Superscripts refer to Ballesteros-Weinstein numbering. Trp-5357.55 is absolutely conserved in class F GPCRs and maps to the intracellular tip of TM VII, a region structurally homologous to the NPXXY motif in class A GPCRs (79, 80). Trp-5357.55 overlays with the Tyr7.53 of the NPXXY motif, which undergoes rearrangement in inactive versus active structures of class A GPCRs (60, 81, 82), Leu-4125.51 is highly conserved across class F GPCRs and also appears in a conformationally labile region of GPCRs. In class A GPCRs, residue 5.51 is one of a group of conserved hydrophobic and aromatic residues (3.40, 5.51, 6.44, 6.48) thought to constitute a “transmission switch” that rearranges when agonist binds (45, 83). Collectively, these constitutively active mutants bolster the notion that Smo cycles through canonical GPCR inactive-active states.

Vismodegib is a Smo inhibitor that binds to the 7TM pocket (Fig. 4) and has been approved for the treatment of advanced BCC. Resistance to Vismodegib usually appears within a few months, however (84). Cancers with active Hh signaling are often driven by inactivating Ptc mutations, but resistance mutations often appear in Smo, the target of the drug. The Vismodegib resistance mutation originally found in medulloblastoma, D473H (55), disrupts Vismodegib binding to Smo but does not result in Smo activation or loss of Smo regulation by physiological levels of Ptc. Additional drug resistance mutations in Smo were found in a mouse model of medulloblastoma where treatment with NVP-LDE225, a Smo 7TM antagonist, led to resistance mutations in Smo that predominantly localize to the 7TM-binding pocket and result in phenotypes similar to D473H (85). Several unique Smo resistance mutations (W281L2.57, V321M3.32) were also recently found in BCC after treatment with Vismodegib (86). W281L2.57 localizes to the base of the 7TM-binding pocket within 3.7 Å of the base of the LY2940680 ligand. V321M3.32 is further buried at the base of the binding pocket and 5.8 Å from SANT1 at its closest point. It is not known whether these mutations function to disrupt binding of Vismodegib to Smo or to activate Smo, but its position in the Smo structure suggests that W281L is more likely to interfere with ligand binding than V321M. Given the rapid resistance to drugs targeting the Smo 7TM pocket, antagonists that bind the Smo CRD hold out the hope that drugs targeting the CRD may prove more effective or less susceptible to resistance when used either alone or in combination with compounds targeting the Smo 7TM pocket (62, 63).

Any discussion of the Smo 7TM and CRD regions naturally leads to questions concerning how these components interact and how their interplay affects the Smo C-terminal tail. Little is known about the structure of the Smo C-tail alone or with the Smo 7TM bundle, but its low complexity and high hydrophilicity suggest that it does not adopt a rigid globular structure. The Smo C-tail is phosphorylated in response to pathway activation, although the identities of the kinases responsible for phosphorylation differ between vertebrates and invertebrates (31, 87). A conformational change of the Drosophila Smo C-tail has been proposed to stem from C-tail phosphorylation altering interactions between positively charged clusters of Arg residues and negatively charged clusters of Asp residues (32), but the verte-
brate Smo C-tail does not possess the Arg clusters. A C-tail conformational change in vertebrates has also been proposed, however (88).

**Conclusion**

Multiple inputs (oxysterol binding to the CRD, small molecule binding to the 7TM pocket, and sterols within the cell membrane) are all capable of modulating Smo activity and presumably conformation. Sorting out what the endogenous inputs are, which of these inputs are important in specific instances, how multiple inputs are integrated, how best to exploit various ways of modulating Smo for anticancer therapies, and the role of Ptc in modulating these inputs present exciting challenges. Recent results have helped clarify the nature and sites of these inputs, however, and provided a framework for understanding how each of the parts fit together to achieve remarkable biological results.

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