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Review

G-protein-coupled receptors, Hedgehog signaling and primary cilia

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ABSTRACT

The Hedgehog (Hh) pathway has become an important model to study the cell biology of primary cilia, and reciprocally, the study of ciliary processes provides an opportunity to solve longstanding mysteries in the mechanism of vertebrate Hh signal transduction. The cilium is emerging as an unique compartment for G-protein-coupled receptor (GPCR) signaling in many systems. Two members of the GPCR family, Smoothened and Gpr161, play important roles in the Hh pathway. We review the current understanding of how these proteins may function to regulate Hh signaling and also highlight some of the critical unanswered questions being tackled by the field. Uncovering GPCR-regulated mechanisms important in Hh signaling may provide therapeutic strategies against the Hh pathway that plays important roles in development, regeneration and cancer.

Contents

1. Introduction

The unexpected discovery that vertebrate Hedgehog (Hh) signaling is dependent on primary cilia a decade ago has had a profound impact on our understanding of this key signaling pathway in development and disease [1]. Primary cilia function as compartments for Hh signaling, with transduction of the signal driven by a set of choreographed protein trafficking events. Indeed, nearly all events in Hh signaling prior to target gene transcription have been linked to ciliary mechanisms. In the absence of signaling, Patched 1 (Ptch1), a 12-pass transmembrane protein that receives Hh ligands along with co-receptors [2,3], is concentrated in and around primary cilia [4]. In this OFF state, Protein Kinase A (PKA) and Suppressor of Fused (SuFu) restrain the activity of the Gli family of transcription factors and promote the formation of truncated Gli repressors (GliR) [5–9]. The orphan rhodopsin family G-protein-coupled receptor (GPCR) Gpr161 localizes to cilia and promotes

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the PKA-mediated generation of GliR [10]. Reception of Hh ligands, such as Sonic Hedgehog (Shh), causes the displacement of Ptch1 and Gpr161 away from the primary cilium [2,8]. This allows accumulation of Smoothened (Smo), a member of the frizzled (class F) family of GPCRs, to high levels in the ciliary membrane [11]. Smo concentration at cilia ultimately leads to activation of the Gli family of transcription factors. Activated Smo has to overcome two negative regulators, SuFu and PKA. Smo signaling promotes the transport of Gli–SuFu complexes to the tips of cilia, allowing Glis to dissociate from SuFu and enter the nucleus to transcribe target genes [12–15]. Ciliary mechanisms are likely critical to understanding the following unsolved mysteries in vertebrate Hh signaling: (a) how is PKA activity regulated at cilia during Gli processing, (b) how is Smo regulated by Ptch1, and (c) how does Smo transmit the signals to the Gli family of transcription factors. A challenge going forward is to understand the biochemical mechanisms that regulate each of these signaling steps at cilia and to understand how these mechanisms are integrated with the dynamic trafficking changes that have been revealed by protein localization studies. We discuss the emerging view that the cilium serves as a unique platform for GPCR signaling with an emphasis on its roles in the Hh pathway. We focus on regulatory mechanisms both upstream and downstream of these two GPCRs, Smo and Gpr161, in the context of their localization and functioning in cilia. We also discuss mechanisms that link cellular GPCR-generated signaling to the transcriptional output of the Hh pathway in different tissues and in the pathophysiology of Hh-dependent cancers.

2. Mechanisms underlying Smoothened activity in primary cilia

2.1. Regulation of Smoothened by Patched 1

An enduring mystery in Hh signaling in all animals revolves around the mechanism by which Ptch1 regulates Smo. Notably, the Ptch1-Smo interaction is the most frequently damaged step in two Hh-driven cancers, medulloblastoma (MB) and basal cell carcinoma (BCC). Current models propose that Ptch1 regulates Smo activity by modulating the concentration or localization of a (yet to be discovered) small molecule ligand. This conclusion has been derived from the following observations: Ptch1 can inhibit Smo catalytically rather than stoichiometrically [16], Ptch1 and Smo do not physically interact in conventional biochemical assays, and Ptch1 demonstrates distant homology to the bacterial Resistance, Nodulation, Division (RND) family of small-molecule pumps [17].

The activity of Smo itself is subject to regulation by a bewildering diversity of synthetic and endogenous small molecules. The plant alkaloid cyclopamine was the first described direct antagonist of Smo and subsequently became the inspiration for a class of anti-Hh cancer drugs that are now in clinical use [18–21]. A number of small molecule screens have since uncovered both direct Smo agonists and antagonists [22–26]. While the endogenous Smo ligand is unknown, sterol-related molecules have been proposed to have a role in Smo activation. In vertebrates, pharmacological or genetic depletion of cholesterol from cells blocks both ligand-induced Hh signaling and constitutive Smo signaling in *Ptch1*$^{-/-}$ cells [19,27,28]. Oxysterols, a class of enigmatic oxidized cholesterol derivatives, were discovered to be potent activators of Hh signaling in multiple systems [29,30]. While an initial study suggested that oxysterols could not be Smo agonists [30], subsequent mechanistic analysis convincingly demonstrated that oxysterols were direct ligands and allosteric modulators of Smo [31]. Surprisingly, detailed pharmacological analysis showed that oxysterols likely bound to a site that was physically distinct from the cyclopamine binding site that had been the focus of research and therapeutic intervention for the prior decade [31]. Finally, Vitamin D3 and derivative analogs have been implicated as Smo antagonists [32]. Interestingly, glucocorticoids, which share a tetracyclic ring skeleton with sterols, have also been identified as synthetic Smo ligands [33].

How do these molecules influence Smo activity? Smo is composed of an N-terminal, extracellular Cysteine-Rich Domain (CRD), homologous to the CRD of the Frizzled (Fz) proteins that bind to Wnt ligands [34]. A linker connects the CRD to the membrane-spanning 7-helix bundle (7TM), which in turn is followed by a cytoplasmic tail. Structures of both the isolated CRD and the isolated 7-helix bundle have been solved and provided views of two distinct ligand binding sites on Smo [35–37]. Liganded structures show that the 7TM bundle, associated extracellular loops, and the CRD linker comprise the "cyclopamine-binding site," which engages ligands that compete with cyclopamine for binding to Smo [35,38]. Oxysterols, on the other hand, bind to the CRD in a hydrophobic groove that corresponds to the groove used by the Fz CRD to bind to the palmitoleyl moiety of Wnt ligands [36,37,39,40]. While present on physically separable domains in Smo, pharmacological studies show that the oxysterol- and cyclopamine-binding sites are allosterically linked [31]. While a Smo structure containing both the CRD and 7TM segments is not yet available, one possibility is that the CRD can influence the 7TM site by interacting with the loops that form the extracellular end of the 7-helix bundle. A Smo molecule lacking the CRD or containing mutations in the 7TM site can still be inhibited by cholesterol depletion, suggesting that the effect of cholesterol may be mediated through a completely distinct mechanism or site on Smo [40]. In this regard, cholesterol-binding sites with regulatory potential have been identified within the transmembrane segments of GPCRs, channels, and transporters (reviewed in [41]).

Despite this progress, the binding site on Smo regulated by Ptch1 remains to be identified. Mutations in the 7TM segment that abrogate the binding of several 7TM ligands have little influence on the ability of Smo to be regulated by Shh, and thus by Ptch1 [40,42]. While some point mutations in the CRD can significantly reduce Shh responsiveness, other mutations that completely abrogate oxysterol binding have no effect [36,39]. Moreover, a truncated Smo molecule lacking the CRD can still be repressed by overproduced Ptch1 and remains weakly responsive to Shh [39,40]. Taken together, these data show that neither of these two sites can be entirely responsible for mediating the inhibitory effect of Ptch1 on Smo.

In addition to ligand-mediated regulation, changes in the levels of Ptch1 and Smo in the ciliary membrane seem to be critical for Smo activation. Ptch1 and Smo undergo a characteristic reciprocal change in localization at the ciliary membrane when signaling is initiated [4,11]. Without Hh ligands, Ptch1 is localized in punctate structures along the length of the ciliary membrane and is found in vesicles around the ciliary base, while Smo is present at low levels. When cells are exposed to Hh, Ptch1 is cleared from cilia and instead Smo accumulates to high levels in the ciliary membrane [4].

There is consensus that the accumulation of Smo in the ciliary membrane is required for downstream signaling. For instance, *Drosophila* Smo, which is normally inactive and not localized in cilia in vertebrate cells, can activate Hh signaling when recruited to cilia by replacing its C-terminal tail with that of vertebrate Smo [39]. While cilia localization may be required for Hh signaling, it is not sufficient. First, Smo appears to be cycling through the cilium even in the absence of Hh ligands. Genetic [43,44] or pharmacological blockade [45] of the retrograde intraflagellar transport (IFT) motor dynein 2, which mediates transport of cargoes from the tip to the base of cilia, leads to Smo accumulation in the ciliary membrane without triggering signaling. Also, certain Smo antagonists, such as cyclopamine, can themselves induce Smo accumulation in cilia without triggering downstream signaling [46]. Finally, the loss of IFT25, an intraflagellar transport protein (a subunit of the IFT

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