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Detergent-solubilized Patched purified from Sf9 cells fails to interact strongly with cognate Hedgehog or Ihog homologs

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ABSTRACT

Patched (Ptc) is a twelve-pass transmembrane protein that functions as a receptor for the Hedgehog (Hh) family of morphogens. In addition to Ptc, several accessory proteins including the CDO/Ihog family of co-receptors are necessary for proper Hh signaling. Structures of Hh proteins bound to members of the CDO/Ihog family are known, but the nature of the full Hh receptor complex is not well understood. We have expressed the *Drosophila* Patched and Mouse Patched-1 proteins in Sf9 cells and find that Sonic Hedgehog will bind to Mouse Patched-1 in isolated Sf9 cell membranes but that purified, detergent-solubilized Ptc proteins do not interact strongly with cognate Hh and CDO/Ihog homologs. These results may reflect a nonnative conformation of detergent-solubilized Ptc or that an additional factor or factors lost during purification are required for high-affinity Ptc binding to Hh.

Introduction

The Hedgehog (Hh)[1] signaling pathway mediates key tissue patterning events during animal development, and abnormal pathway activity is associated with several cancers [1,2]. Hh proteins are secreted morphogens that specify cell fates in neighboring tissues in a concentration-dependent manner [3,4]. The twelve-pass transmembrane protein Patched (Ptc) has been identified as a key Hh receptor in genetic [5–8] and cell-based binding studies [9–11]. In the absence of Hh, Ptc constitutively inhibits the activity of Smoothened (Smo) [6,12], a seven-pass transmembrane protein. The mechanism of this inhibition is unknown but does not appear to involve a direct interaction between Ptc and Smo [12]. In the presence of Hh, this inhibition is relieved and the pathway is activated. For recent reviews, see [13–16].

Many additional proteins modulate Hh pathway activity, but their presence and activity are not always conserved across phyla. For instance, Gas1 positively regulates Hh signaling in mammals [17], but no Gas1 homolog exists in the fruit fly. Hhip, a cell surface protein that acts as a negative regulator by binding and sequestering Hh proteins in vertebrates [18] also has no apparent ortholog in the fly. The mammalian proteins CDO and BOC are orthologous to the fly proteins Ihog and Boi and each binds its cognate Hh protein, but the manner and co-factor dependence of Hh binding by fly and mammalian orthologs is not conserved [19].

Despite the central importance of the Hh signaling pathway in animal development and the identification of many key pathway components, little is known about the molecular details connecting these components. Ptc is presumed to control Smo activity by transporting a small sterol-like molecule, a hypothesis based on an array of circumstantial evidence: the homology of Ptc to proton antiporters in the RND superfamily; the presence in Ptc of a sterol-sensing domain, which in other eukaryotic homologs is related to cholesterol trafficking; the indirect inhibition of Smo by Ptc, which suggests an intermediate [12]; and recent structural and biochemical studies of Smo showing that sterols bind Smo and modulate its activity [20–23]. Nevertheless, ligand transport by Ptc has not been conclusively demonstrated, nor has a physiological ligand for Smo been identified. More generally, how Hh proteins modulate the activity of Ptc is not known, and no functional assay for purified Ptc has been established.

A direct interaction between Hh and Ptc is clearly the simplest and most likely interpretation for high-affinity Hh binding to Ptc-expressing cells and Hh modulation of Ptc activity [9,11]. Assays measuring binding to the cell surface leave open the possibility that other cellular factors could be involved, however. For example, most cell-based binding studies predated knowledge of the importance of accessory proteins Gas1, CDO/Ihog and BOC/Boi for mediating interactions between Hh and the cell surface. These proteins are essential for both fly [24] and mammalian Hh signaling [17,25].

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[1] *Abbreviations used:* Hh, Hedgehog; Ptc, Patched; Smo, Smoothened; Shh, Sonic Hedgehog; DH, Decasaccharide; HMT-, His-Myc-TEV-; SBP, Streptavidin Binding Peptide; HBM, Honeybee Mellitin; SEC, exclusion chromatography; CD, Circular Dichroism; DH, decaheparin.

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Members of the CDO family of proteins bind Hh proteins directly with low micromolar affinities [26,19,27], whereas the affinity of mammalian Sonic Hedgehog (Shh) for binding to the surface of Ptc-expressing cells is in the low nanomolar range [9,11]. This difference in affinity, as well as the fact that high-affinity cell-surface binding is dependent on the presence of Ptc, would seem to indicate that Ptc itself directly binds Shh but that the affinity for the binary Shh–Ptc interaction may be weaker than the affinity of Hh for Ptc-expressing cells. The role of the CDO/Ihog family of proteins appears to be to function as co-receptors that enhance binding affinity. In this case, a ternary complex between Ptc, Hh and members of the CDO/Ihog family may represent the initial signaling complex at the cell surface. Evidence for such ternary complexes has been found for *Drosophila* Ptc and Ihog [24], but the evidence in the mammalian pathway is contradictory. Although CDO, BOC or Gas1 appear to be required along with Ptc for normal signaling in mammals [17,25], the addition of the soluble Hh-binding domain of CDO (CDOFn3) actually competes for ShhN binding to Ptc on the cell surface [19]. This observation suggests that the binding surfaces for Ptc and CDO on ShhN may overlap. This binding competition has been rationalized with the positive role of both Ptc and CDO in Hh signaling by the observation that physiological Hh is found in multivalent particles [28,29], allowing simultaneous Ptc and CDO binding. Multivalency is not required for high-affinity binding of Shh to Ptc on cells, however, as monomeric ShhN expressed in *E. coli* binds Ptc-expressing cells with high affinity [9,11]. Hh proteins also bind sulfated glycosaminoglycans with affinities that can be in the micromolar range [29,30], which also likely contributes to high-affinity interactions with the cell surface.

A major barrier to understanding Ptc activity and the nature of its interactions with Hh pathway components has been the difficulty of isolating functional forms of Ptc or Ptc fragments. Most information on the reported molecular mechanisms and binding partners of Ptc has been obtained indirectly, using cell, tissue or whole animal-based studies. We therefore undertook to express and purify Mouse and *Drosophila* Ptc proteins with intact transmembrane and extracellular regions for binding studies with Hh and other Hh pathway components. Sonic Hedgehog will bind to Mouse Ptc in isolated Sf9 cell membranes, but we surprisingly find that Ptc proteins extracted and purified in the presence of detergents do not interact with soluble, cognate Hh or CDO/Ihog homologs with high affinity as either binary or ternary complexes.

Experimental procedures

Materials

All chemicals were purchased from Sigma unless otherwise noted. Detergents were purchased from Affymetrix and included n-dodecyl-β-D-maltopyranoside (DDM), n-dodecylphosphocholine (fos-choline 12, FC-12), and 2,2-di decylpropane-1,3-bis-β-D-maltopyranoside (lauryl maltose neopentyl glycol, LMNG). Low molecular weight heparin (LMWH, average molecular weight 3000 Da, sodium salt, from porcine sources) was purchased from P212121.com. Heparin Decasaccharide (DH) was purchased from Neoparin Inc. Antibodies for western blotting were mouse α-Myc monoclonal (9E10), which was isolated from hybridoma growth medium; rabbit α-Ihog polyclonal and mouse α-Hh monoclonal antibodies, which were gifts from P. Beachy; and appropriate HRP-conjugated secondary antibodies.

Buffers

Ni^{2+} Binding Buffer consisted of 35 mM NaH_2PO_4, 300 mM NaCl, and 15 mM imidazole adjusted to pH 8.0 with NaOH at 25 °C. Ni^{2+} Wash Buffer consisted of 10 mM Tris base, 10 mM Tris HCl, and 300 mM NaCl. Ni^{2+} Elution Buffer was Ni^{2+} Wash Buffer with 250 mM imidazole. Strep Wash Buffer was 20 mM MOPS, 10 mM NaOH, and 200 mM NaCl. Strep Elution Buffer was Strep Wash Buffer with 2.5 mM desthiobiotin. Pull-Down Buffer was Strep Wash Buffer containing 0.01% LMNG and 0.2 mM TCEP. CD Buffer was 10 mM NaH_2PO_4, 150 mM NaF, titrated to pH 7.2 with NaOH. CPM Thermal Stability Buffer was 20 mM HEPES pH 7.5, 200 mM NaCl, 0.02% LMNG.

Proteins and expression vectors

IhogFn1, IhogFn2, IhogFn12 and IhogFn12ΔH are the first, second, or both Type III Fibronectin (FnIII) domains from Ihog, respectively, with ΔH referring to an Ihog surface mutant with reduced heparin binding [26]. BOCFn3, BOCFn23 and BOCFn13 are FnIII domains 3, 2–3, and 1–3 respectively from BOC. These BOC and Ihog fragments were expressed as His-Myc-TEV- (HMT-) fusion proteins using the vector pT7HMT [31]. HhN, ShhN and ShhN-SC (The "Surface C" mutant of ShhN [11], which is deficient in binding to Ptc-expressing cells) were subcloned into a modified version of pMAL-c2x as described [19]. ShhFL and HhFL are the full-length Mouse and *Drosophila* Hh proteins including native signal sequences and C-terminal self-splicing domains. IhogFn12TM consisted of the native Ihog signal sequence followed by the first and second FnIII domains and the transmembrane region, but with the intracellular region truncated. Ski and Hhat are the entire native Hh acyltransferases from *Drosophila* and Mouse, respectively. MmHhip consisted of the β-propeller and following two EGF domains of Mouse Hhip. *Drosophila* Patched and Mouse Patched-1 proteins are the tagged Mouse and *Drosophila* Ptc proteins with their C-termini truncated immediately after the final predicted trans membrane helix (DmPtcT1 and MmPtcT1, respectively). See Supplementary Table 1 for detailed protein sequence specifications.

DmPtcT1 and MmPtcT1 proteins were expressed fused to a concatenated series of N-terminal tags including the Streptavidin Binding Peptide (SBP) [32], an HRV3C protease site, a 6 × His tag, a Myc tag, and a TEV protease site. Tags and Ptc proteins were cloned into the transfer vector pFastBac1 (Invitrogen). For co-expression of ShhFL with Hhat, HhFL with Ski, and DmPtcT1 with IhogFn12TM, transfer vectors were constructed from pFastBacDual (Invitrogen). Hhip was cloned into a modified pFastBac1 vector containing the Honeybee Mellitin (HBM) signal sequence to target for secretion, followed by 8 × His, SBP, Myc and TEV sequences.

Production of recombinant baculoviruses

Recombinant bacmids and baculoviruses for insect cell expression were constructed using the Bac-to-Bac system (Invitrogen) following manufacturer's instructions. After transfecting Sf9 cells with bacmid DNA, the secreted virus (designated P1) was amplified two more times (P2 and P3 viruses) following manufacturer's instructions. P3 virus was used for protein production.

Bacterial expression and purification of HhN, IhogFn1, IhogFn2, IhogFn12, IhogFn12ΔH, ShhN, ShhN-SC, BOCFn3, BOCFn23 and BOCFn13

All expression plasmids were transformed into BL21 and plated on LB with appropriate antibiotics. Single colonies were picked, grown overnight, and used to inoculate TB in baffled flasks at 225 RPM and 37 °C. Bacteria were grown to an optical density (600 nm) of around 0.8, at which point the incubator temperature was lowered to 16 °C and the cultures allowed to shake for an additional hour. IPTG was then added to a final concentration of