Current Biology, Vol. 12, R437–R439, June 25, 2002, ©2002 Elsevier Science Ltd. All rights reserved. PII S0960-9822(02)00920-X

Hedgehog Signalling: Pulling Apart Patched and Smoothened

Dispatch

David R. Stark

Two integral membrane proteins, Patched and Smoothened, were for a long time thought to comprise a preformed receptor complex for secreted Hedgehog signalling proteins. Recent analyses of the subcellular distribution of these proteins argue strongly against this simple model.

Signalling proteins of the Hedgehog (Hh) family are essential for patterning and morphogenesis in most multicellular organisms [1]. Reception of the Hh signal at the cell surface requires the activity of two transmembrane proteins. Patched (Ptc) is a polytopic protein predicted to contain twelve transmembrane domains and binds directly to Hh proteins and inhibits the activity of a second component of the receptor complex, Smoothened (Smo). Smo is a seven-pass membrane protein that is required to transduce the Hh signal. In the absence of Ptc, Smo activity is independent of the Hh ligand and constitutively activates the Hh signalling pathway [2]. Thus the binding of Hh to Ptc is thought to liberate Smo from repression by Ptc. A simple model had been proposed whereby Ptc inhibits Smo via a direct, stable, physical interaction, and Hh binding to Ptc causes a conformational change in this Ptc–Smo receptor complex which frees Smo from inhibition. A number of recent studies, including one in this issue of *Current Biology*, put forward compelling arguments against this model [3–7].

A Ptc–Smo Complex

Two key papers [8,9] showed that epitope-tagged vertebrate homologues of Ptc and Smo could be coimmunoprecipitated from cells expressing them at high levels, in the presence and absence of a Hh signal, and that a trimeric complex of Ptc–Smo–Hh could be detected after cell-surface crosslinking [8]. Although significant levels of the bimolecular complex were present in cells in which a Hh reporter gene was strongly activated, these results did not decisively show the composition of the active receptor complex. It was proposed that Hh binding might evoke dissociation of a preformed Ptc–Smo complex freeing active Smo, or a conformational change within the stable complex to activate Smo.

To understand how the activities of Ptc and Smo are regulated by Hh, recent studies have addressed the distribution of Hh, Ptc and Smo in cultured *Drosophila* cells, as well as *in vivo*. [3–5,7]. These studies present evidence that Smo protein is not present in a complex bound to Ptc and suggest that in the absence of Hh, Ptc indirectly modifies Smo protein to generate an inactive moiety, in a non-stochiometric reaction [4,5].

Current Biology, Elsevier Science London, 84 Theobald's Road, London, WC1X 8RR, UK.
E-mail: david.stark@current-biology.com

Where are Ptc and Smo?

A key observation is that Smo protein levels increase dramatically in cells responding to Hh. Denef and colleagues [4] looked in detail at the subcellular distribution of Smo and Ptc in cultured *Drosophila* cells and in the cells of larval wing discs. Their results produced two further conclusions: that the binding of Hh to Ptc at the cell surface leads to the removal of Ptc protein from the plasma membrane; and that the activation of the Hh signal transduction pathway triggers the phosphorylation of Smo concomitant with a dramatic increase in Smo at the cell surface. In a parsimonious interpretation of their data, the authors invoke a requirement for at least an additional kinase/phosphatase module in the transduction pathway. Thus, in the absence of Hh, Ptc's constitutive inhibitory activity might be mediated by the action of a phosphatase to counter the constitutive phosphorylation of Smo protein by an appropriate kinase.

A Putative Role for Intracellular Trafficking

Ptc protein contains a so-called sterol-sensing domain (SSD), a motif found in proteins that regulate cholesterol metabolism, and the sorting and recycling of cholesterol and glycosphingolipids in the late endosome–lysosome system [10]. In addition, it is possible to block both the response of cells to Hh and the intracellular transport of cholesterol by a number of compounds including the plant steroidal alkaloids cyclopamine and jervine [11]. However, cells expressing mutant forms of NPC1 that block cholesterol transport do not have a diminished response to Hh signalling [12], suggesting that these compounds instead affect a vesicular transport route that is shared by Hh signalling components and cholesterol transport.

A more recent study in the mouse [13] has shown that mutations in the Ras-like GTPase Rab23, important for vesicle fusion during endocytosis and exocytosis, inhibit Hh signalling in the developing neural tube. These mutations appear to lead to the ligand-independent activation of the Hh pathway, further emphasising the role of intracellular trafficking in the response to Hh. Together these data strongly suggest that the intracellular trafficking of Ptc and Smo through the late endosome–lysosome system is pivotal for regulation of the Hh signalling pathway.

Subcellular Localisation of Ptc

In two *Current Biology* papers published last year, the Ingham [5] and Guerrero [6] labs published detailed mutational analyses of the mechanism by which Ptc regulates Smo activity. Ingham and colleagues [5] described the subcellular distributions of Ptc and Hh

in embryos homozygous for a number of Ptc loss-of-function mutations whose sequence was already known, or determined by this group. The authors confirmed the accumulation of wild-type Ptc in multivesicular bodies and endosomes, and showed that some of these vesicles contain Hh. Importantly, even cells some distance from a source of Hh also contained punctate accumulations of Ptc protein, suggesting that Ptc cycles between endosomes and the cell surface even in the absence of Hh. Of the Ptc mutants used in this study, three were distributed similarly to the wild-type protein and, strikingly, they colocalised with Hh protein in a punctate distribution. Thus these mutant proteins appear to bind and endocytose Hh protein, yet fail to inhibit Smo activity.

Ptc's SSD: Hh Sequestration and Smo Inhibition

Interestingly, two of these three mutations affected the SSD of Ptc. In the study by the Guerrero lab [6], a Ptc mutant was engineered to contain a single amino acid substitution in the SSD. The mutation behaved as a strong loss-of-function allele, yet the distribution of the mutant protein was unaffected by this change; nor did it alter the ability of Ptc to bind and sequester Hh. As noted above, the occurrence of an SSD in Ptc has been argued by some to support a role for the protein in interacting in some way with cholesterol. However, it has been far from clear whether this cholesterol is, for example, the moiety added to the active amino-terminal fragment of Hh during its biogenesis or the cholesterol of putative lipid microdomains in specific vesicles such as late endosomes which might be important for targeting Ptc for degradation [4,10].

In light of the data suggesting that Ptc regulates Smo activity by affecting its post-translational modification or stability, Ingham and colleagues [5] and Guerrero and colleagues [6] both suggest that the SSD plays a pivotal role in the ability of Ptc to regulate Smo. And Ingham and colleagues suggest that Ptc might be acting in a similar manner to other SSD-containing proteins, such as SCAP and NPC1, to regulate the intracellular trafficking of Smo to a compartment where it is targeted for modification or degradation.

Ptc and Smo Co-internalisation

In this issue of *Current Biology*, Roelink and colleagues [3] present the most detailed analysis to date of the subcellular localisation of Ptc and Smo and show how this distribution is affected by a Hh ligand. The authors generated lines of KNRK cells stably expressing epitope-tagged vertebrate homologues of Ptc and Smo, alone and in combination. Their results clearly show by confocal microscopy that, when expressed in the absence of Smo, Ptc is constitutively internalised and degraded in lysosomes. Internalisation of Ptc is significantly increased when these cells are exposed to the biologically active amino-terminal fragment of Sonic hedgehog (Shh). When Ptc and Smo are expressed together in the same cells, reflecting more closely the physiological scenario, in the absence of Shh, the rate of Ptc internalisation is greatly reduced. Furthermore, Ptc and Smo can be seen to be internalised together and enter the endosomal pathway, albeit relatively slowly, and are degraded in lysosomes.

Hh Induces Segregation of Ptc and Smo

Exposure of cells expressing Ptc and Smo to Shh leads to the co-internalisation of Ptc, Smo and Shh, but now only Ptc and Shh are degraded in lysosomes. The authors show that, after entering late endosomes together, Smo is somehow segregated from the Ptc–Shh complex, and is now presumably free to signal and return to the cell surface. The accumulation of Smo at the cell surface in response to Shh protein is reported here, confirming similar data from previous studies [4,6]. In support of this late endosome segregation model, the authors also show that antibodies to the late-endosome-specific lipid lysobisphosphatidic acid (LBPA), an endosomal membrane component essential for endosome structure and function, are able to interfere with the segregation of Ptc and Smo and inhibit activation of the pathway.

Importantly, these antibodies will also inhibit Shh signalling in chick neural plate explants, suggesting that the intracellular movement of Ptc and Smo in these KRNK cell lines is physiologically meaningful. In addition the authors look at the distribution of constitutively active mutant Smo proteins, previously isolated from sporadic basal cell carcinomas [14] that are not inhibited by Ptc protein [9]. The distribution of these mutant proteins, M2 and to a lesser extent M1, is consistent with them failing to colocalise and cointernalise with Ptc. Hence the Hh-dependent segregation of these molecules from Ptc is circumvented and the pathway is activated independently of a Hh signal.

Do Ptc and Smo Interact Directly?

The results of Roelink and colleagues [3] illustrate the dynamic nature of Ptc and Smo localisation, and highlight the intimate relationship of these proteins throughout most compartments. One significant result that Roelink and others (for example [7]) have failed to confirm is the coimmunoprecipitation of Ptc and Smo. The idea of a preformed receptor complex comprising Smo and Ptc was an attractive, mechanistically simple interpretation of the early genetic analyses of Ptc and Smo function, supported by limited biochemical data. However, these early studies used transiently transfected cells expressing very high levels of protein. And although epitope-tagged molecules were expressed, the forms of Smo protein detected by subsequent western blotting ran at unusually high molecular weights. Subsequent studies mimicking more physiological conditions have failed to detect a stable Ptc–Smo complex, and no study has shown such a complex to be present *in vivo*.

Future Directions

The results of Roelink *et al.* [3] clearly strengthen support for the model whereby Ptc indirectly regulates Smo activity; a key event is the segregation of Smo from 'activated' Ptc in late endosomes. However, these results raise a number of questions. Why is Smo transported into the late endocytic pathway along with Ptc? How does Hh binding to Ptc trigger

the segregation of Smo? Why should SSD mutants of Ptc behave as dominant-negative molecules [5,6]? And at a more basic level, just what is the physiological relevance of activated Smo being transported to the cell surface?

Resolving the mysteries over the missing biological activities of these molecules is a priority. Is Smo a G-protein coupled receptor (see for example [15,16])? Is Ptc a transmembrane permease like NPC1 (see [10,17])? And of course, tracking down the putative missing components of the pathway might also help provide some eagerly awaited answers.

References

1. Ingham, P.W. and McMahon, A.P. (2001). Hedgehog signaling in animal development: paradigms and principles. Genes Dev. *15*, 3059–3087.
2. Ingham P.W. (1998). Transducing Hedgehog: the story so far. EMBO J. *17*; 3505–3511.
3. Incardona., J.P., Gruenberg, J., and Roelink, H. (2002). Sonic hedgehog induces the segregation of Patched and Smoothened in endosomes. Curr. Biol. this issue.
4. Denef, N., Neubuser., D, Perez, L., and Cohen, S.M. (2000). Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened. Cell *102*, 521–531.
5. Strutt, H., Thomas, C., Nakano, Y., Stark, D., Neave, B., Taylor, A.M., and Ingham, P.W. (2000). Mutations in the sterol-sensing domain of Patched suggest a role for vesicular trafficking in Smoothened regulation. Curr. Biol. *11*, 608–613.
6. Martin, V., Carrillo, G., Torroja, C., and Guerrero, I. (2000). The sterol-sensing domain of Patched protein seems to control Smoothened activity through Patched vesicular trafficking. Curr. Biol. *11*, 601–607.
7. Alcedo, J., Zou, Y., and Noll M. (2000). Posttranscriptional regulation of smoothened is part of a self-correcting mechanism in the Hedgehog signaling system. Mol. Cell *6*, 457–465.
8. Stone, D.,M., Hynes, M., Armanini, M., Swanson T.A, Gu, Q., Johnson, R.L., Scott, M.P., Pennica, D., *et al.* (1996). The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. Nature *384*, 129–134.
9. Murone, M., Rosenthal., A., de Sauvage, F.J., (1999). Mutants of cubitus interruptus that are independent of PKA regulation are independent of hedgehog signaling. Curr. Biol. *9*, 76–84.
10. Kuwabara, P.E., and Labouesse, M. (2002). The sterol-sensing domain: multiple families, a unique role? Trends in Genet. *18*, 193–201.
11. Cooper, M.K., Porter, J.A., Young, K.E., Beachy, P.A. (1998). Teratogen-mediated inhibition of target tissue response to Shh signaling. Science *280*, 1603–1607.
12. Incardona, J.P., Gaffield, W., Lange, Y., Cooney, A., Pentchev, P.G., Liu, S., Watson, J.A., Kapur, R.P., and Roelink, H. (2000). Cyclopamine inhibition of Sonic hedgehog signal transduction is not mediated through effects on cholesterol transport. Dev. Biol. *224*, 440–452.
13. Eggenschwiler, J.T., Espinoza, E., and Anderson, K.V. (2002). Rab23 is an essential negative regulator of the mouse Sonic hedgehog signalling pathway. Naure *412*, 194–197.
14. Xie, J., Murone, M., Luoh, S.M., Ryan, A., Gu, Q., Zhang, C., Bonifas, J.M., Lam, C.W., *et al*. (1998). Activating Smoothened mutations in sporadic basal-cell carcinoma. Nature *391*, 90–92.
15. Taipale, J., Chen, J.K., Cooper, M.K., Wang, B., Mann, R.K., Milenkovic, L., Scott, M.P., and Beachy, P.A. (2000). Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. Nature *406*, 1005–1009.
16. DeCamp, D.L., Thompson, T.M., de Sauvage F.J., and Lerner, M.R. (2000). Smoothened activates Gαi-mediated signaling in frog melanophores. J. Chem. Biol. *275*, 26322–26327.
17. Davies, J.P., Chen, F.W., Ioannou, Y.A. (2000). Transmembrane molecular pump activity of Niemann-Pick C1 protein. Science *290*, 2295–2298.