The tumour-suppressor gene *patched* encodes a candidate receptor for Sonic hedgehog

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The protein Sonic hedgehog (Shh) controls patterning and growth during vertebrate development. Here we demonstrate that it binds Patched (vPtc), which has been identified as a tumour-suppressor protein in basal cell carcinoma, with high affinity. We show that Ptc can form a physical complex with a newly cloned vertebrate homologue of the *Drosophila* protein Smoothened (vSmo), and that vSmo is coexpressed with vPtc in many tissues but does not bind Shh directly. These findings, combined with available genetic evidence from *Drosophila*, support the hypothesis that Ptc is a receptor for Shh, and that vSmo could be a signalling component that is linked to Ptc.

THE vertebrate homologues of the *Drosophila* segment polarity gene *hedgehog* (hh) compose a family of at least five proteins[1–6]. The most notable of these is Sonic hedgehog (Shh), which is expressed in multiple embryonic tissues including the ventral forebrain, notochord, floorplate, limb bud and hindgut endoderm. Shh seems to be involved in the specification of multiple ventral cell types including motor[7,8], serotonergic[9] (M.H. and A.R., unpublished results), dopaminergic[10] and forebrain[11] neurons in the neural tube, and in the formation of sclerotome, vertebra and ribs[12,13]. In the embryonic limb bud, Shh is likely to be the signal required for anterior posterior patterning[3] and for the establishment of a functional apical ectodermal ridge[14,15], whereas in the hindgut Shh produced by the endoderm has been implicated in the specification of visceral hindgut mesoderm[16]. The critical role of Shh in tissue patterning is further illustrated by the finding that mice deficient in Shh die before birth and display multiple developmental defects including cyclopia, absence of ventral cells in the neural tube, absence of the spinal column and most of the ribs, absence of distal limb structures, and degeneration of the notochord[17].

Other members of the Hedgehog protein family have also been implicated in tissue patterning. For example, Desert hedgehog (Dhh) was shown to be essential for testes development[18], Indian hedgehog (Ihh) is required for chondrocyte differentiation[19], and zebra-fish-specific *Echidna* and Tiggy-winkle hedgehog are involved in muscle-cell specification[3] and eye patterning[6], respectively.

Despite the importance of Hedgehog proteins in vertebrate development, little is known about their receptors or mechanism of action. Genetic evidence in *Drosophila* implicated two proteins as candidate receptors for Hh: Patched (Ptc)[20–23] a putative 12 transmembrane (TM) protein; and Smoothened (Smo)[24–27], a 7 TM protein. Ptc is thought to be a negative regulator of Hh, and the Hh signalling cascade seems to be constitutively active in its absence. In contrast, Smo is an essential component in the Hh pathway, and Smo mutants display the same phenotype as Hh mutants.

Recently, vertebrate homologues of Ptc (but not of Smo) have been identified in the chick[28], mouse[29] and human[30,31]. Furthermore, the human homologue of Ptc (hPtc) was found to be mutated or inactivated in the basal cell naevus syndrome (BCNS), a familial complex of cancers and developmental abnormalities, and in basal cell carcinoma (BCC), leading to the proposal that hPtc is a tumour-suppressor gene[30,31].

Given the physiological and clinical importance of the vertebrate Hh, we attempted to identify receptor candidates for these molecules.

Isolation of a vertebrate Smo homologue

Studies in *Drosophila* suggested that Smo is a receptor for Hh[26,27], so we began by searching for vertebrate homologues of Smo. A complementary DNA library, generated from Shh-responsive, embryonic day (E) 9–10 rat tissues, was screened at low stringency with a *Drosophila* Smo (dSmo) probe, and overlapping cDNAs were isolated which encoded a protein of 794 amino acids. Subsequently, a human Smo homologue, which is 94% identical to the rat Smo, was identified. The human and rat Smo proteins (hSmo and rSmo) are 33% homologous to dSmo (homology in the transmembrane domains is 50%), 23% homologous to the *Drosophila* Wingless receptor[32], and 25% homologous to the vertebrate Frizzled protein[33]. Like their *Drosophila* homologue[26,27], hSmo and rSmo appear to be 7 TM G-protein-coupled receptors[34], possessing four glycosylation sites and a putative extracellular amino terminus 203–205 amino acids long, which

includes 13 stereotypically spaced cysteines and could bind a polypeptide ligand (Fig. 1).

Tissue distribution of the vertebrate Smo

Examination of mouse tissues by northern blot analysis using rSmo as a probe demonstrated the presence of an approximately 4.4-kilobase *Smo* transcript from embryonic day-7 onwards (Fig. 2*a*). *In situ* hybridization analysis further revealed that the rat *smo* messenger RNA was found in Shh-responsive tissues, such as the early neural folds and neural tube[1,2,4,7,8,10], pre-somitic mesoderm and somites[12,13], and developing limb bud[3], gut[16] and eye[26]. Transcripts for *rsmo* were also observed in those tissues with development regulated by other members of the vertebrate Hh protein family, such as embryonic testes (Dhh)[18], cartilage (Ihh)[19] and muscle (the zebrafish *Echidna* Hh)[5] (Fig. 2*b* and data not shown). In all of these tissues, the temporal and spatial distribution of rSmo and rPtc shows considerable overlap. For example, in the embryonic nervous system, *rsmo* and *rptc* are initially expressed throughout the neural folds and early neural tube (*rsmo* mRNA is evenly distributed along the dorsal–ventral axis, whereas *rptc* mRNA is found at higher levels ventrally) (Fig. 2*b*, E9, E10). By E12, expression of both *rsmo* and *rptc* declines dramatically in lateral parts of the neural tube, and by E15 their mRNAs are restricted to cells which are in close proximity to the ventricular zone (Fig. 2*b*, E12, E15). The *rsmo* and *rptc* mRNAs are also found adjacent to Shh-expressing cells in the embryonic lung, epiglottis,

FIG. 1 The primary structure of hSmo and rSmo, and their homology to dSmo. Signal peptide sequences are underlined, conserved amino acids are boxed, cysteines are marked with asterisks, potential glycosylation sites are marked with dashed boxes, and the 7 hydrophobic transmembrane domains are shaded.

thymus, tongue, jaw, taste buds and teeth, as well as in other tissues that do not contain high levels of *Shh* mRNA, such as the skin (Fig. 2*b*; compare top panels of Shh, Smo and Ptc). In adults, *smo* mRNA was found in multiple tissues including heart, brain, liver, lung, skeletal muscle, kidney and testis (Fig. 2*a*).

Shh does not bind Smo

To characterize a possible physical association between Shh and rSmo, competition-binding, cross-linking and co-immunoprecipitation experiments were performed. Surprisingly, given the receptor-like structure of rSmo and the genetic evidence that dSmo is a receptor for *Drosophila* Hh[26,27], no direct interaction between mouse Shh and rSmo could be detected. Specifically, we did not detect any binding of IgG-Shh-N chimaeric protein (the N-terminal biologically active portion of Shh[35] fused in-frame to the Fc portion (the crystallizable fragment) of human IgG-γ1) (Fig. 3*b*), or of epitope-tagged Shh or Dhh (data not shown) to cells that expressed rSmo (Fig. 3*a*). Similarly, ^{125}I-Shh-N could not be co-immunoprecipitated by antibodies against an epitope-tagged rSmo (Fig. 4*c*), nor could the IgG–Shh-N chimaeric protein immunoprecipitate rSmo (Fig. 4*a*). Finally, no cross-linking or equilibrium binding between ^{125}I-Shh-N and rSmo could be demonstrated (Fig. 4*b* and data not shown), and no binding of tagged *Drosophila* Hh to rSmo or to dSmo could be detected (data not shown).

Shh binds vPtc with high affinity

Although we cannot rule out the possibility that additional vertebrate homologues of dSmo exist which may bind Shh directly, our findings argue against the hypothesis that rSmo, acting alone, is a receptor for Shh or Dhh. However, the possibility remained that rSmo might be an essential component of a putative Shh receptor complex, of which the ligand-binding function is provided by another protein. We therefore examined whether vPtc, the only other transmembrane protein known to be associated with the Hh signal cascade[20–23,28,29], could be a ligand-binding component of such a receptor complex. Indeed, we found that epitope-tagged Shh-N, as well as IgG-Shh-N, bind specifically and saturably to 293 cells expressing the mouse Ptc (mPtc; mPtc is 33% identical to its *Drosophila* counterpart) (Fig. 3*c–e* and data not shown). (Preliminary experiments suggest that *Drosophila* Hh also binds to mPtc.) Furthermore, mPtc was immunoprecipitated by IgG–Shh-N (Fig. 4*a*), and antibodies to an epitope-tagged mPtc readily co-immunoprecipitated ^{125}I-Shh-N (Fig. 4*c*). Finally, competition-binding experiments demonstrated that ^{125}I-Shh-N could be crosslinked to mPtc (Fig. 4*b*), and that the two proteins interacted with a K_d of 460 pM (Fig. 4*d*). No binding of ^{125}I-Shh-N to parental 293 cells or to cells expressing exogenous rSmo could be detected (data not shown), and no change in the affinity between Shh-N and Ptc was observed in the presence of transfected rSmo (Fig. 4*d*). However, given the wide tissue distribution of Smo (Fig. 2), we cannot exclude the possibility that low levels of

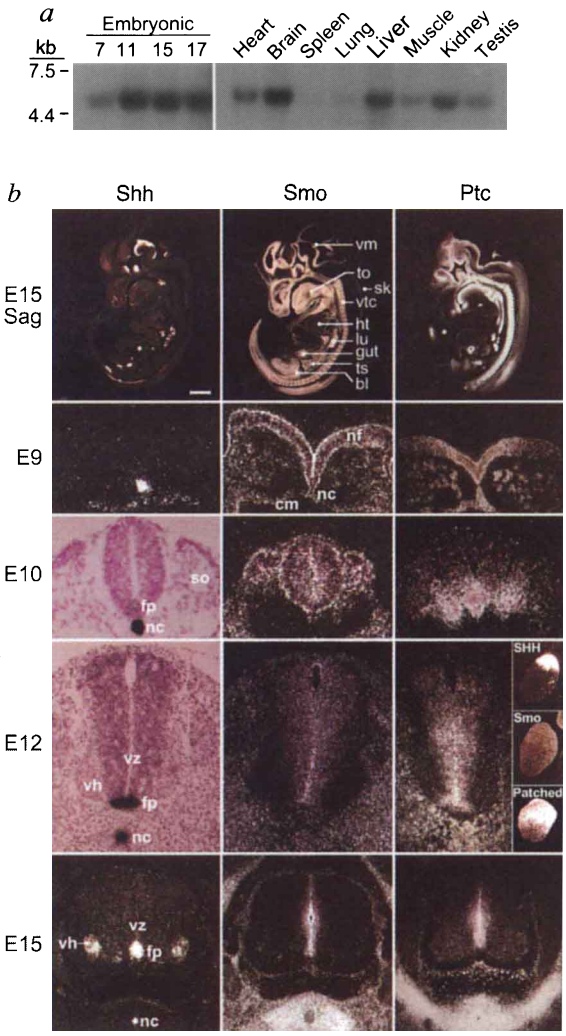

FIG. 2 Tissue distribution of Shh, rSmo and rPtc. *a*, Northern blot analysis of mouse and rat tissues with rSmo probe. *b*, *In situ* hybridization of Shh (left), rSmo (middle) and rPtc (right, not including insets) to rat tissues. Row E15 Sag, sagittal sections through E15 rat embryos; rows E9, E10, E12 and E15, coronal sections through E9 neural folds, E10 neural tube and somites, and E12 and E15 neural tube. Insets in row E12 show sections through forelimb bud of E12 rat embryos. Abbreviations: bl, bladder; cm, cardiac mesoderm; fp, floorplate; ht, heart; lu, lung; nc, notochord; nf, neural fold; sk, skin; so, somite; to, tongue; ts, testes; vh, ventral horn; vm, ventral midbrain; vtc, vertebral column; vz, ventricular zone. Scale bar shown in top left panel is 700 μm in row E15 Sag; 50 μm in E9; 45 μm in E10; 40 μm in E12; 140 mm in E15; and 720 mm in insets.

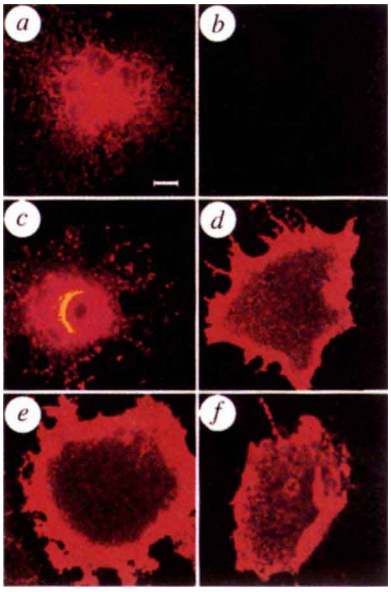

FIG. 3 Shh-N and Dhh-N bind to cells expressing mPtc but not to cells expressing rSmo. Staining of COS-7 cells expressing the Flag-tagged rSmo (*a*, *b*) or Myc-tagged mPtc (*c–f*), with anti-Flag (Smo) antibody (*a*), anti-Myc (mPtc) antibody (*c*), IgG-Shh-N (*b*, *d*), a Flag-tagged Shh-N (*e*), a Flag-tagged Dhh-N (*f*). Only cells expressing mPtc bind the various tagged forms of Shh-N and Dhh-N. No binding of other IgG fusion proteins to mPtc-expressing cells and no binding of the various tagged forms of Shh-N to untransfected cells were detected (data not shown). Scale bar, approximately 5 μm.

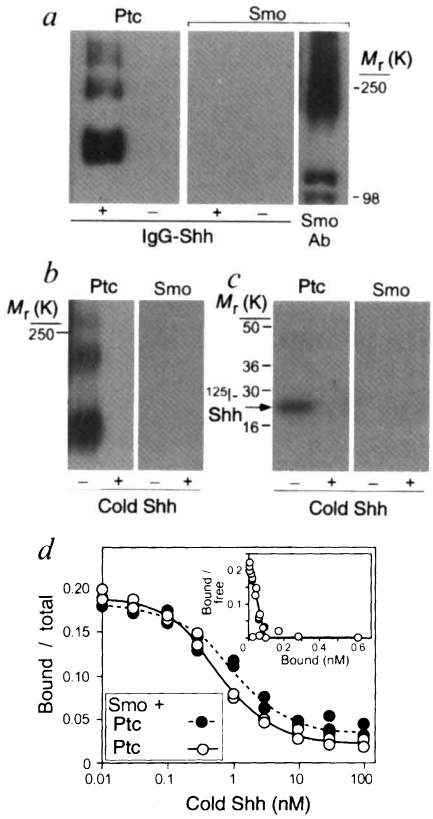

FIG. 4 Shh-N binds to mPtc but not to rSmo. *a*, Co-immunoprecipitation of epitope-tagged mPtc (Ptc) or epitope-tagged rSmo (Smo) with IgG-Shh-N (IgG-Shh), from cells expressing either mPtc or rSmo alone, as indicated. Only mPtc could be co-immunoprecipitated by the IgG-Shh-N protein. Immunoprecipitation of rSmo could be achieved only with antibodies to the Smo epitope tag (Smo Ab). *b*, Cross-linking of ^{125}I-Shh-N (^{125}I-Shh) to cells expressing mPtc or rSmo in the absence or presence of excess unlabelled Shh-N (Cold Shh—or +). ^{125}I-Shh-N could be cross-linked only to mPtc-expressing cells. *c*, Co-immunoprecipitation of ^{125}I-Shh-N by antibodies to Myc-tagged mPtc (Ptc) or to Flag–tagged rSmo (Smo). ^{125}I-Shh-N could be co-immunoprecipitated with antibodies to the Myc-tagged mPtc, but not with antibodies to the Flag-tagged rSmo, and only in cells that expressed mPtc. *d*, Competition binding of purified recombinant murine ^{125}I-Shh-N to cells expressing mPtc or mPtc + rSmo. The Scatchard analysis (inset) gave a K_d value of 460 pM. No binding to cells expressing rSmo alone was observed (not shown), and there was no increase in binding affinity to mPtc in the presence of rSmo. Labels above photographs: Ptc, cells transfected with a Myc-tagged mPtc alone; Smo, cells transfected with a Flag-tagged rSmo alone.

endogenous Smo expressed in 293 cells contributed to the Shh-N-Ptc interaction. In addition, we find that Shh is not the only member of the Hh protein family that binds Ptc: another member of the Hh protein family, Dhh also binds specifically to cells expressing Ptc (Fig. 3*f*).

Ptc, Smo and Shh form a physical complex

We next determined whether Ptc and Smo are present in a co-immunoprecipitable protein complex. Human embryonic kidney (293) cells were transiently transfected with expression vectors for epitope-tagged rSmo (Flag epitope) and mPtc (Myc epitope), cells were lysed, and solubilized protein complexes were immunoprecipitated with the antibody to one of the epitopes and then analysed on a western blot using the antibody to the other. In cells expressing mPtc or rSmo alone, no protein complexes were detected. Similarly, in cells that expressed mPtc together with either a gD-tagged transmembrane tyrosine kinase receptor, trkB[36], a flag-tagged 7 TM receptor for interleukin-8 (ref. 37) or a flag-tagged rat Frizzled[33], no co-immunoprecipitation of Ptc was observed when lysates were immunoprecipitated with antibodies to the various epitope tags (Fig. 5*d*, *e* and data not shown). In contrast, in cells expressing both mPtc and rSmo (Fig. 5*a*), rSmo was readily co-immunoprecipitated by antibodies against the epitope-tagged mPtc (Fig. 5*b*), and mPtc was readily co-immunoprecipitated by antibodies against the epitope-tagged rSmo (Fig. 5*c*) (approximately 30% of the immunoprecipitable rSmo and mPtc were found in a complex). Further, ^{125}I-Shh-N could be co-immunoprecipitated by antibodies against either the epitope-tagged rSmo or mPtc, from cells expressing both rSmo and mPtc, but not from cells expressing rSmo alone (Fig. 5*f*, *g*). These

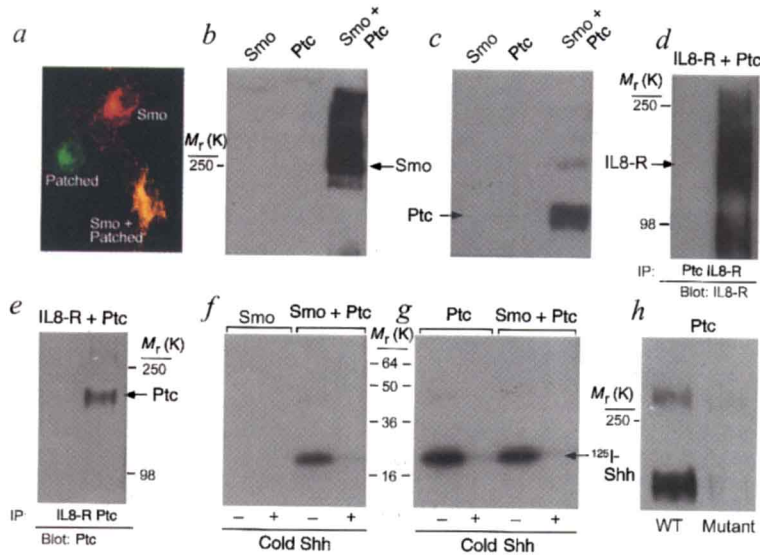

FIG. 5 Ptc and Smo can form a protein complex. *a*, Double immunohistochemical staining of mPtc (green) and rSmo (red) in transfected cells. Yellow indicates coexpression of the two proteins. *b*, *c*, Detection of mPtc–rSmo complex by co-immunoprecipitation from dual-transfected cells. *b*, Immunoprecipitation with antibodies to the Myc-tagged mPtc and analysis on a western blot with antibodies to Flag-tagged rSmo. *c*, Immunoprecipitatin with antibodies to the Flag-tagged rSmo and analysis on a western blot with antibodies to Myc-tagged mPtc. Protein complexes containing both rSmo and mPtc are readily detected in both cases. *d*, *e*, Immunoprecipitation with antibodies to the Flag-tagged interleukin-8 receptor (IL8-R) or to Myc-tagged mPtc from cells expressing both flag-tagged IL8-R and Myc-tagged mPtc, and analysis on a western blot with anti-Flag antibody (IL8-R) (*d*) or anti-Myc antibody (mPtc) (*e*). *f*, *g*, Co-immunoprecipitation of ^{125}I-Shh-N bound to cells expressing rSmo and mPtc or mPtc alone, with antibodies to either rSmo (*f*) or mPtc (*g*) epitope tags. Protein complexes containing both rSmo and ^{125}I-Shh-N are detected only in cells that express both mPtc and rSmo. *h*, Western blot showing the transient expression level of a Myc-tagged, wild-type (WT) or mutated Ptc protein in 293 cells; the mutant retained a complete open reading frame (insertion of amino acids Pro-Asn-Ile after amino acid 815) (ref. 31). The level of the mutated Ptc protein (Mutant) was less than its wild-type counterpart. Labels above photographs: Ptc, cells transfected with a Myc-tagged mPtc alone; Smo, cells transfected with a Flag-tagged rSmo alone; Smo + Ptc, cells transfected with expression vectors for both proteins; IL8-R + Ptc, cells transfected with expression vectors for both Flag-tagged IL8-R and Myc-tagged mPtc.

findings are consistent with the idea that Shh-N, rSmo and mPtc are present in the same protein complex, and that a Smo-Shh-N complex does not form in the absence of Ptc.

Discussion

We have provided evidence that mPtc binds Shh-N with high affinity; that mPtc and rSmo are co-expressed in multiple tissues; and that these two proteins can form a complex to which Shh-N binds. We have not demonstrated that rSmo indeed functions in the Shh pathway in a manner similar to its *Drosophila* counterpart[26,27], nor have we excluded the possibility that vPtc can transduce signals independent of vSmo, or that Shh-N uses additional receptors. With these limitations in mind, our findings, combined with previous genetic studies in *Drosophila*[26,27] and human[30,31], are best explained by the hypothesis that vPtc is a ligand-binding component, and vSmo a signalling component, in a multi-subunit Shh receptor complex (Fig. 6). In other multi-component receptor systems, the ligand-binding subunits often act as activators[38,39], but here, as previously postulated[23] and as our data confirm, the Hh-binding protein (vPtc) appears to be a ligand-regulated suppressor of a signalling unit (vSmo) (Fig. 6). It remains to be determined whether Smo is constitutively active in the absence of Ptc or whether, under these circumstances, Smo would still require a specific ligand for activation.

Genetic mutations leading to a truncated[30,31] or unstable (Fig. 5*h*) Ptc protein appear to be associated with familial and sporadic forms of BCC. This correlation, combined with the fact that Ptc is a high-affinity binding protein for Shh, suggests that the Hh system may provide mitogenic or differentiative signals to basal cells in the skin throughout life. Furthermore, our findings raise the possibility that BCNS and BCC might result from constitutive activation of vSmo, which becomes oncogenic after its release from inhibition by Ptc (Fig. 6).

Notably, Shh is not the only Hh protein that interacts directly with Ptc. As we have demonstrated, at least one other member of this protein family (Dhh) can bind Ptc. These results suggest that multiple Hh proteins can mediate their distinct functions through a single receptor system.

With a receptor for Shh (and Dhh) identified, it will now be possible to study the mechanism by which the Hh family exerts mitogenic, differentiative and morphogenic effects at the molecular level. Furthermore, the identification of Smo as a candidate oncogene in familial and sporadic BCCs may lead to a better understanding of, and eventually to a therapy for, this common human cancer. □

Methods

Cloning of the rat Smo homologue. The rat Smo homologue (rSmo) was identified by screening a rat E9–10 cDNA library at low stringency with a probe encompassing the entire coding region of *Drosophila smoothened*[26]. Eight positive plaques were identified, and three overlapping cDNA clones were sequenced. cDNAs for the human homologue of Smo (hSmo) were isolated from a human embryonic lung library (Clontech), using the rat cDNA as probe.

Northern blot analysis and *in situ* hybridization. For northern analysis, mouse embryonic and rat multiple tissue northern blots (Clontech) were used. For *in situ* hybridization, E9–15.5 rat embryos were immersion-fixed overnight at 4 °C in 4% paraformaldehyde and cryoprotected overnight in 20% sucrose. P1 rat brains were frozen fresh. All tissues were sectioned at 16 μm, and processed for *in situ* hybridization using ^{33}P-UTP labelled RNA probes[39].

Expression constructs and immunohistochemistry. cDNAs for the various proteins were cloned into a cytomegalovirus-based expression vector, and epitope tags added to the extreme C terminus by PCR-based mutagenesis. For mPtc cDNA, the Myc epitope tag was added after amino acid 1293, with an engineered stop codon immediately following. This resulted in a protein partially truncated at the C terminus. Experiments with the full-length *mptc* demonstrated similar Shh binding (data not shown). The *ptc* mutant was generated by site-directed mutagenesis of the Myc-tagged *mptc* cDNA. For visualization of protein expression, COS-7 cells, transiently transfected with expression constructs, were stained using anti-Flag M2 (IBI) or anti-Myc antibodies permeabilized and (Invitrogen or A-14; Santa Cruz), followed by Cy3-conjugated anti-mouse IgG (Jackson Immunoresearch) and/or bodily-conjugated anti-rabbit IgG (Molecular Probes). For visualization of Shh binding, COS-7 cells transiently expressing rSmo or mPtc were exposed to tagged Shh or Dhh (2 h at 4 °C), fixed, and stained with a Cy3-conjugated anti-human IgG (for IgG-Shh-N) or anti-Flag antibody (for Flag-tagged Shh-N or Dhh-N) followed by Cy-3 conjugated anti-mouse IgG.

Protein production. Human embryonic kidney 293 cells were transiently transfected with expression vectors encoding either IgG-Shh-N (Shh-N fused in frame after amino acid 198 to the Fc portion of human IgG-γ1), flag-tagged Shh-N, or flag-tagged Dhh-N (the flag epitope was added immediately after the internal proteolytic cleavage site) and medium was collected after 48 h. Mouse Shh-N was produced and purified as described[10].

Immunoprecipitation, competitive binding and cross-linking analyses. Embryonic kidney 293 cells transiently transfected with either Flag-tagged rSmo or Myc-tagged mPtc were used in all of the above experiments. For the co-immunoprecipitation, cells were incubated in the presence or absence of the IgG-Shh-N chimaera (1 μg ml^{-1}, 30 min at 37 °C), or in the presence of ^{125}I-Shh-N with or without an excess of unlabelled Shh-N (2 h at 4 °C) and then lysed[38]. Lysates were centrifuged and soluble protein complexes were immunoprecipitated with either protein A–Sepharose (for the IgG-Shh-N), or anti-Flag or anti-Myc antibodies followed by protein A–Sepharose (for epitope-tagged rSmo or mPtc, respectively), and then separated on a denatured 8% SDS polyacrylamide gel. Proteins were detected either by exposure of the dried gel to film (for ^{125}I-Shh-N) or by blotting to nitrocellulose and probing with antibodies to Flag or Myc epitopes, using the ECL detection system (Amersham). To examine the interaction between rSmo and mPtc, cells were either lysed directly or after exposure to ^{125}I-Shh-N with or without an excess of unlabelled Shh-N (30 min at 37 °C, followed by 3 washes in phosphate buffered saline). Lysates were immunoprecipitated with antibodies against one of the epitope tags, subject to denaturing SDS–PAGE, and then resulting gels were either exposed to film (when ^{125}I-Shh-N was present) or transferred to nitrocellulose and analysed with antibody against the second epitope tag. For cross-linking, cells were resuspended in buffer containing 50 pM ^{125}I-Shh-N with or without a 1,000-fold excess of unlabelled Shh-N, and incubated for 2 h at 4 °C. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (10 mM) and *N*-hydroxysulphosuccinimide (5 mM) (Pierce) were added (30 min at room temperature), and the cells were washed 3 times with PBS. Cells were then lysed and protein complexes were immunoprecipitated with antibodies to the epitope tags as indicated, and run on a 4% SDS polyacrylamide gel. For equilibrium binding analysis, 293 cells were incubated with 50 pM ^{125}I-Shh-N and various concentrations of unlabelled Shh-N (Cold Ligand). The IGOR program was used to determine K_d.

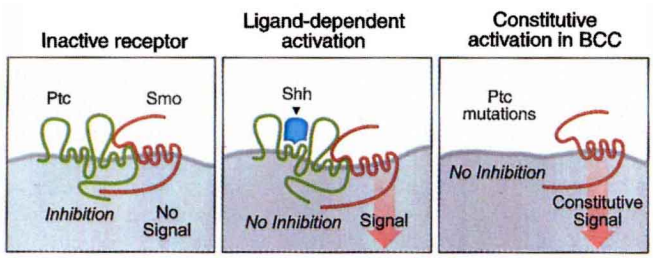

FIG. 6 Model describing the putative Shh receptor and its activation by Shh-N or following inactivation of Ptc. (Note that the actual binding site of Shh-N to Ptc and the interaction sites between Ptc and Smo have not been determined at the molecular level. In addition, the illustrated secondary structures of Smo and Ptc are based only on the primary structure of these proteins and are therefore hypothetical.)

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