Human Receptors Patched and Smoothened Partially Transduce Hedgehog Signal When Expressed in Drosophila Cells*§

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In humans, dysfunctions of the Hedgehog receptors Patched and Smoothened are responsible for numerous pathologies. However, signaling mechanisms involving these receptors are less well characterized in mammals than in Drosophila. To obtain structure-function relationship information on human Patched and Smoothened, we expressed these human receptors in Schneider 2 cells. We show here that, as its Drosophila counterpart, human Patched is able to repress the signaling pathway in the absence of Hedgehog ligand. In response to Hedgehog, human Patched is able to release Drosophila Smoothened inhibition, suggesting that human Patched is expressed in a functional state in Drosophila cells. We also provide experiments showing that human Smo, when expressed in Schneider cells, is able to bind the alkaloid cyclopamine, suggesting that it is expressed in a native conformational state. Furthermore, contrary to Drosophila Smoothened, human Smoothened does not interact with the kinesin Costal 2 and thus is unable to transduce the Hedgehog signal. Moreover, cell surface fluorescent labeling suggest that human Smoothened is enriched at the Schneider 2 plasma membrane in response to Hedgehog. These results suggest that human Smoothened is expressed in a functional state in Drosophila cells, where it undergoes a regulation of its localization comparable with its Drosophila homologue. Thus, we propose that the upstream part of the Hedgehog pathway involving Hedgehog interaction with Patched, regulation of Smoothened by Patched, and Smoothened enrichment at the plasma membrane is highly conserved between Drosophila and humans; in contrast, signaling downstream of Smoothened is different.

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§1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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By comparing the genomes of flies and humans, Rubin et al. (1) have demonstrated that 61% of the genes involved in human diseases have orthologues in the fly, and, in particular, 68% of cancer genes are found to have fly orthologues. This is the case of the Hedgehog (Hh)§ pathway, essential for patterning and morphogenesis, where the major players were first identified by genetic screens in Drosophila (2–5). Aberrant Hh pathway activity plays a pathological role in the growth of a group of endoderm-derived tumors that together account for 25% of human cancer death (6–8). Moreover, recent studies suggest that dysfunction of the Hh pathway in stem or precursor cells might contribute to tumorigenesis and neurodegenerative disorders (9–11). The Hh peptide, which is dually modified at its N and C termini by palmitoyl and cholesterol adducts, respectively, triggers the pathway activation by stoichiometric binding to Patched (Ptc), a 12-transmembrane segment (TMS) protein (4, 12). Ptc is an atypical receptor, the activity of which is repressed upon ligand binding. In Drosophila, this results in the stabilization of the seven-TMS protein Smoothened (Smo) at the plasma membrane, which in turn interacts with a cytoplasmic complex and activates the cytoplasmic transcription factor Cubitus interruptus (Ci) (13). It has been shown that the kinesin Costal 2 (Cos2), component of a large cytoplasmic complex, can bind to Smo and that this interaction is important for Ci activation (14–16). In contrast, transfected Smo in mammalian cultured cells is internalized after activation of the pathway instead of accumulating at the cell surface (17). Similarly, the internalization of Smo has been observed when the pathway is activated using a Hh agonist and can be reversed by treatment with the Hh antagonist cyclopamine (18). These findings suggested that Smo localization might be regulated differently in flies and mammals. However, recent data on the role of cilia in the Hh pathway suggest that vertebrate Smo, like the Drosophila protein, is recruited to specialized membranes in response to ligand (19, 20). In the absence of Hh, Ptc was proposed to act catalytically to suppress Smo activation (21). This Hh signal

§ The abbreviations used are: Hh, Hedgehog; Ptc, Patched; TMS, transmembrane segment; Smo, Smoothened; Ci, Cubitus interruptus; Cos2, Costal 2; dsRNAi, double-stranded RNA interference; MAP, multitag affinity purification; hSmo, human Smo; dSmo, Drosophila Smo; Fu, Fused; hPtc, human Ptc; dPtc, Drosophila Ptc.
response scheme is globally conserved from insects to mammals. However, the molecular mechanism of Smo inhibition by Ptc is unknown, and signaling downstream of Smo, if relatively well understood in Drosophila, still needs to be resolved in mammals (13, 22).

Dysfunction of Ptc and Smo are responsible of numerous human pathologies, making these receptors interesting therapeutic targets. In order to obtain a sufficient amount of Ptc and Smo to purify them and determine their three-dimensional structure, we expressed the human Ptc and Smo in Drosophila Schneider 2 cells. The functional characterization of the human receptors expressed in Schneider 2 cells using double-stranded RNA interference (dsRNAi) against Drosophila Ptc and Smo, fluorescence labeling, and immunoprecipitation experiments strongly suggest that human receptors Ptc and Smo are functionally expressed in Schneider 2 cells. Based on the results presented in this paper and on sequence alignment analyses, we discuss the evolution of the Hh pathway between Drosophila and humans.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Vectors**—For expression in Drosophila Schneider 2 cells, we used the pAc5.1/V5-His type A (pAc) and pMT/V5-His (pMT) vectors (Invitrogen) containing the strong constitutive Ac5 actin promoter and the heavy metal inducible MT metallothionein promoter, respectively. The multitag affinity purification (MAP) (23) sequence was inserted into the KpnI and BamHI restriction sites of pAc and into the XbaI and BamHI restriction sites of pMT, suppressing the V5 into the MAP (23) sequence was inserted into the strong constitutive Ac5 actin promoter and the heavy metal inducible MT metallothionein promoter, respectively.

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**Membrane Protein Preparation and Purification**—All steps were performed at 4 °C. Cells were collected, centrifuged, washed two times in phosphate-buffered saline and one time in H2O, and resuspended in hypotonic buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and protease inhibitor mixture (Roche Applied Science). After 10 min in ice, cells were broken by passages through a syringe. Cellular remains were pelleted at 430 × g for 10 min, and supernatant was centrifuged for 30 min at 20,000 × g to collect heavy membranes, essentially plasma membranes. Membrane proteins were resuspended in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, protease inhibitor mixture, and 20% glycerol. Solubilization and purification were performed as already described by De Rivoyre et al. (23).

**Western Blot Analysis**—Cells were collected, pelleted, and then resuspended in a lysis buffer containing 1% Triton, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitor mixture. Membrane proteins were resuspended and then loaded onto SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (Hybond-C Extra; Amersham Biosciences). Following a blocking step in 5% nonfat dried milk in TBS, cells were incubated overnight in MTBST supplemented with polyclonal rabbit anti-Cos2 (1:5000), rabbit anti-Fused (F) (1:1000), rabbit anti-dSmo (1:200; (14)), anti-Myc (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or monoclonal mouse anti-HA (1:200) antibodies. Membrane was washed three times in TBS-T and then incubated in MTBST supplemented with corresponding IgG horseradish peroxidase-coupled for 1 h. Membrane was washed three times with TBS-T and revealed using ECL reagents.

**Double-stranded RNA Interference in S2 Cells**—dsRNAi was produced by transcription in vitro with T7 polymerase on PCR products corresponding to amino acids 740–970 of DpTc and amino acids 141–370 of DsMo. Transfection with dsRNAi into S2 cells was performed as described by Ruel et al. (14). Briefly, 37 μg of dsRNAi per million cells were incubated in 1 ml of culture media without serum and then thoroughly agitated for 1 min and then completed with 2 ml of culture media containing serum after 3 days of incubation, allowing protein expression turnover. Transfected cells were then split into control and Hh-containing medium and incubated for an additional 16 h. Samples were prepared for analysis by SDS-PAGE and immunoblotting.
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Surface Immunofluorescent Labeling—Stably hSmo-expressing cells or S2 cells transfected with 0.5 µg of pAc-hSmo-MAP were incubated or not with Hh-conditioned medium, and cell surface labeling was performed as described by Kurhara et al. (25). Cells were incubated for 1 h at 0 °C with a rabbit polyclonal antibody raised against amino acids 488–787 mapping at both intra- and extracellular C terminus domain of human Smo (1:100; N-300; Santa Cruz Biotechnology) in S2 medium containing 10% of fetal bovine serum. After dilution and centrifugation, cells were incubated for 30 min at 0 °C with a rhodamine fluorescent secondary antibody (Alexa 568 goat anti-rabbit IgG (1:500; Molecular Probes)) in S2 medium containing 10% of fetal bovine serum. After dilution and centrifugation, cells were resuspended in phosphate-buffered saline plus 1% formaldehyde. Rhodamine fluorescence was analyzed by flow cytometry (FACSScan; BD Biosciences), and fluorescence microscopy with image acquisition was performed using a confocal system (Zeiss LSM 510 Meta) with an objective Plan Apochromat ×63/1.4 oil differential interference contrast, and an Applied Precision Deltavision System (Applied Precision, Issaquah, WA) built on an Olympus IX 70 base and a ×40/1.35 Uapo objective at 1024 × 1024 pixel resolution. Cell fluorescence was analyzed using Image J software.

Cycloamine Binding—Cycloamine binding assays were performed using the fluorescent derivative BODIPY-cycloamine generously provided by P. Beachy and adapted from Ref. 26. Cells that were wild-type, stably hSmo-expressing, or transiently transfected with 0.5 µg of pAc-hSmo-MAP S2, treated or not with Hh conditioned medium, were incubated with 5 or 50 nM of BODIPY-cycloamine for 4 h at 25 °C, collected by centrifugation, and resuspended in phosphate-buffered saline plus 1% formaldehyde. BODIPY fluorescence was analyzed by flow cytometry (FACSScan; BD Biosciences).

Immunoprecipitation—Immunoprecipitation experiments were performed as described by Ruel et al. (14). Briefly, 10 µg of protein G-Sepharose bound to anti-Cos2, anti-HA, or anti-Myc were added to the clarified cell lysates at 4 °C for 2 h, and immunocomplexes were washed five times with lysis buffer. ECL reagents were used for antibody detection after blotting to nitrocellulose membranes.

Protein Quantification—The proteins were quantified using the Bio-Rad protein assay.

RESULTS

Establishment of Schneider Cell Lines Stably Expressing Human Ptc and Human Smo—Because Ptc and Smo are involved in numerous pathologies in humans, they provide interesting therapeutic targets. We decided to overexpress each protein separately in a heterologous system (Schneider cells) in order to purify them to determine their three-dimensional structure. Schneider 2 (S2) cells are suitable hosts for the expression of large amounts of recombinant eukaryotic protein, since they are inducible, nonlytic, stable, and able to reach a cell density as high as 3 × 10⁶ cells/ml (10-fold higher than SF9 cells) (27–29). hPtc and hSmo cDNA were subcloned in S2 cell expression vectors in which the sequence MAP was previously inserted in place of the existent tags V5 and His. This MAP sequence, fused to the hSmo and hPtc C-terminal ends, provides several epitopes to follow hSmo and hPtc expression as well as opportunities for rapid purification under mild conditions using several affinity chromatography columns (23). S2 cells have been co-transfected with pMT-hSmo-MAP, pMT-hPtc-MAP, pAc-hSmo-MAP, or pAc-hPtc-MAP and the pCo-Hygro that allows transformant selection and stable S2 cell line establishment using hygromycin. We observed by immunoblotting using anti-HA antibodies directed against the MAP sequence that hSmo and hPtc were transiently expressed under metallothionein or actin promoter (data not shown). After treatment with hygromycin, we obtained cell populations expressing hPtc or hSmo. Several clonal stable cell lines were then established from each polyclonal population, and the expression levels of hSmo and hPtc were analyzed by Western blot. We observed that expression levels for both proteins were higher under the actin promoter than under the metallothionein one (data not shown). We present in Fig. 1 Western blots using anti-HA antibodies performed on total extracts from different clones expressing hSmo (Fig. 1A) or hPtc (Fig. 1B) under the actin promoter. The five pAc-hSmo-MAP transfected clones present a specific highly immunoreactive signal around 100 kDa, corresponding to the molecular mass calculated from hSmo-MAP sequence (Fig. 1A, lanes 1–5). By comparison with hSmo-MAP expressed in yeast (23), which gives bands around 80 and 100 kDa, corresponding probably to nonglycosylated and glycosylated forms of hSmo, respectively, the 100-kDa band observed here may correspond to a glycosylated form of hSmo-MAP. A high molecular weight-specific band that certainly corresponds to an hSmo oligomer is also detected. A strong signal around 110 kDa is present in all extracts, including those from wild-type S2 cells corresponding to an endogenous protein highly expressed in S2 cells. One of the clones stably

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**FIGURE 1. Human Smo and human Ptc are expressed in S2 cells.** Western blot with anti-HA antibodies on total extract prepared from S2 cells transfected with pAc-hSmo-MAP (A) or pAc-hPtc-MAP (B). A, lane 6, polyclonal pAc-hSmo-MAP cells; A, lanes 1–5, derived clonal pAc-hSmo-MAP cell lines; A, lane 7, wild-type S2 cells. B, lane 1, polyclonal pAc-hPtc-MAP cells; B, lanes 2 and 3, derived clonal pAc-hPtc-MAP cell lines. C, hPtc and hSmo are retained on affinity resins after solubilization. The initial material corresponds to a 20,000 × g membrane preparation enriched for plasma membrane proteins. Shown are 40 µg of membrane protein preparation (lane 1); solubilized fraction (lane 2); flow-through fractions from Ni²⁺-nitrilotriacetic acid (lane 3), calmodulin (lane 4), and streptavidin (lane 5) resins; and fractions retained on Ni²⁺-nitrilotriacetic acid (lane 6), calmodulin (lane 7), and streptavidin (lane 8) resins.
transfected with pAc-hPtc-MAP vector presents a strong signal around 180 kDa, corresponding to the expected molecular weight of recombinant hPtc-MAP protein (Fig. 1B, lane 2). We also observed the presence of minor lower molecular weight bands that probably correspond to degradation products. We selected one pAc-hPtc-MAP clone and one pAc-hSmo-MAP clone for their high expression levels of hPtc and hSmo.

Heavy membrane fractions containing plasma membrane fragments were prepared from a 20-ml culture of hPtc- and hSmo-expressing cell lines selected. Anti-HA immunoblots presented in Fig. 1C show specific signals around 180 kDa for hPtc-expressing cells and 100 kDa for hSmo-expressing cells, indicating that both hPtc and hSmo are expressed at the plasma membrane in the S2 cell lines selected. Membrane fractions were solubilized in buffer containing 1% detergent dodecyl-β-D-maltoside and incubated with 50 μl of Ni²⁺-nitrilotriacetic acid, calmodulin, or streptavidin resins. Fractions eluted from the different resins present a specific anti-HA signal at 180 or 100 kDa, corresponding to hPtc-MAP or hSmo-MAP, respectively (Fig. 1C). A weak band around 110 kDa is also observable in membrane preparations. This band corresponds to the highly expressed contaminant observed in the total extracts presented in Fig. 1, A and B, but is not retained on the affinity resins as shown in the purification experiments performed on membrane preparation from wild-type S2 cells (supplemental Fig. 1). These experiments indicate that the three affinity domains of the MAP sequence fused in the hPtc and hSmo C terminus allow efficient binding on the corresponding resins and that this strategy can be used to purify human Ptc and human Smo, according to our recent study on the expression of hSmo in yeast (23). In order to know if hPtc and hSmo expressed in the S2 cell lines selected are in a functional state, we tested hPtc and hSmo activities by different means.

**Human Ptc Is Able to Replace Drosophila Ptc in the Drosophila Hh Pathway**—S2 cells present the advantage of possessing a functional Hh pathway from Ptc to the Fu/Cos2 cytoplasmic complex, but transcriptional response is lacking due to the absence of Ci (30). In response to Hh protein, Smo is submitted to post-translational modifications leading to its stabilization (Fig. 2A, lane 2). This stabilization is visualized by increased levels of Smo at the plasma membrane, a step that seems to be necessary to induce signal transduction (31–34). Consequently, Fu and Cos2 are phosphorylated (5, 14, 35), as visualized by
their electrophoretic mobility shifts (Fig. 2A, compare lanes 1 and 2). We observe that in the S2 cell line expressing hPtc, Fu and Cos2 behave as in wild-type S2 cells in the absence of Hh (Fig. 2A, lane 5). In response to Hh, Fu and Cos2 phosphorylation and Smo accumulation are observed in this cell line (Fig. 2A, lane 6). One possibility is that the inhibitory effect of hPtc is sensitive to Drosophila Hh. Indeed, we noted that in several experiments Fu and Cos2 phosphorylation and Smo stabilization were not induced as strongly as in wild-type S2 cells, probably due to the high level of hPtc expression and thus to hPtc receptors free of Hh ligand.

dsRNAi, inhibiting the expression of selected proteins, is a very efficient tool for dissecting transduction pathways in S2 cells (15, 36). The effect of dsRNAi on Hh pathway can be measured, analyzing the status of Smo, Fu, and Cos2. As already described (14), upon inhibition of dPtc expression (by dsRNAi directed against Drosophila Ptc (dsRNAi-dPtc)), the dSmo level is increased and Fu and Cos2 are phosphorylated even in the absence of Hh ligand (Fig. 2A, lane 3), underlining the inhibitory effect of Ptc on the Hh pathway. In order to see if the human form of Ptc is able to replace its Drosophila homologue, hPtc-expressing cells were treated with dsRNAi directed against dPtc (dsRNAi-dPtc). Upon expression of hPtc, Fu and Cos2 phosphorylation and dSmo stabilization are not induced by dsRNAi-dPtc treatment (Fig. 2A, lane 7), as if hPtc were able to replace dPtc by repressing the pathway. Incubation of these treated cells with Hh ligand induces phosphorylation of Fu and Cos2 and also accumulation of dSmo (Fig. 2A, lane 8), suggesting that hPtc activity is sensitive to Hh. This experiment has been repeated four times and gave similar results.

Our results indicate that human Ptc expressed in the stable cell line selected is able to replace at least partially the Drosophila Ptc and therefore is expressed in a functional state. To confirm this, hPtc-expressing S2 cells were treated with dsRNAi directed against both dPtc and hPtc. In these conditions, we observe high Fu and Cos2 phosphorylation and dSmo stabilization in the absence of Hh (Fig. 2B, lane 4). This suggests that the strong reduction of Fu and Cos2 phosphorylation and of dSmo accumulation observed in hPtc-expressing cells treated with dsRNAi-dPtc in the absence of Hh (Fig. 2A, lane 7 and B (lane 2)) is probably due to the inhibitory effect of human Ptc on the Drosophila Hh pathway.

We then tested if hSmo could rescue the lack of dSmo in S2 cells. In the absence of dSmo (after treatment of cells with dsRNAi directed against dSmo (dsRNAi-dSmo)), Fu and Cos2 phosphorylation is not induced by Hh (compare lanes 2 and 4 of Fig. 2C) (14). Expression of hSmo can be detected in S2 transfected cells at a similar protein level with or without treatment by Hh (in total cell lysates) (Fig. 2C, lanes 5–8). When this cell line is treated with dsRNAi directed against Drosophila Smo (dsRNAi-dSmo), we do not observe Fu and Cos2 phosphorylation in response to Hh (Fig. 2C, lane 8). This result suggests that human Smo is not able to compensate for the absence of Drosophila Smo. In order to understand the reason why hSmo does not transduce Hh signal and to know if hSmo is expressed in a functional state in S2 cells, we tested hSmo activity by other means.
by confocal microscopy shows that the rhodamine fluorescence at the cell surface of hSmo-expressing cells significantly increases (1.6 times) after Hh treatment (Fig. 4A). In the same way, flow cytometry analyses show that the small population of cells presenting a rhodamine fluorescence observable in the R2 region of each graph increases after Hh treatment in the cell population transfected with hSmo in comparison with the cell population transfected with empty vector (Fig. 4B). Taking into account the weak percentage of fluorescent cells, suggesting that the fraction of antibodies interacting with the last extracellular loop is low, we performed the same experiments with seven independent transfections. The results summarized in Fig. 4C show that despite the very low labeling efficiency, Hh treatment significantly increases the percentage of rhodamine fluorescent cells in R2 for the cell population transfected with hSmo.

Both confocal microscopy and flow cytometry experiments show a significant increase of the hSmo-expressing cell surface labeling after Hh treatment. This suggests that the amount of hSmo at the S2 cell surface is enriched in response to Hh.

Plasma membranes were prepared from cells transiently transfected with 0.5 μg of pAc-hSmo-MAP (hSmo S2) or with empty vector (WT S2), treated or not with Hh. The anti-HA immunoblot presented in Fig. 5 reveals the presence of a low amount of hSmo at the plasma membrane of hSmo-transfected cells before Hh treatment. We estimated from three independent experiments that the amount of hSmo in the plasma membrane preparations is increased by an average of 1.5-fold after Hh.
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A

|            | Wild-type S2 cells | hSmo-expressing S2 cells |
|------------|--------------------|--------------------------|
| Hh         | -                  | +                        |

![Images of fluorescence intensity graphs]

B

|            | Wild-type S2 cells | hSmo-transfected S2 cells |
|------------|--------------------|---------------------------|
| Hh         | -                  | +                         |

![Images of flow cytometry plots]

C

![Graph showing percentage of rhodamine fluorescent cells in S2]

WT S2       hSmo S2
Human Patched and Smoothened Expressed in Drosophila Cells

Human Smo Does Not Interact with Cos2—In Drosophila, upon Hh induction, the kinesin Cos2 interacts with Smo stabilized at the plasma membrane, controls the stability of the Smo-Cos2-Fu complex, and allows Ci activation and transcriptional responses (14, 15, 33). As shown in Fig. 6A, lanes 3 and 4, using antibodies directed against Cos2, dSmo and Fu co-immunoprecipitated with Cos2, and, in Hh-treated cells, the association of dSmo with Cos2 and Fu was enriched in Cos2 immunoprecipitates. In hSmo-expressing cells, we observe that although dSmo co-immunoprecipitated with phosphorylated Cos2 and Fu in the presence of Hh, hSmo did not co-immunoprecipitate with Cos2 or Fu (Fig. 6A, lanes 5 and 6). Similarly, the use of antibodies directed against the hemagglutinin antigen present in the MAP sequence at the C-terminal end of hSmo reveals that neither Cos2 nor Fu co-immunoprecipitates with hSmo (Fig. 6A, lanes 7 and 8). These experiments indicate that in the hSmo-expressing cell line selected, Cos2 interacts with dSmo but not with hSmo. To avoid competition between hSmo and dSmo, we performed an immunoprecipitation of hSmo in dSmo RNAi-treated cells (Fig. 6B). In such cells, although the amount of endogenous dSmo was highly decreased, no interaction was observed between hSmo and the Fu-Cos2 complex.

It has been previously shown that Cos2 interacts with the cytoplasmic tail of dSmo (14, 15). This domain is not well conserved between Drosophila and vertebrate species (38) (Fig. 8). To know if this cytoplasmic tail could be responsible for the absence of association between Cos2 and hSmo, we analyzed the interaction between Cos2 and a mSmo-dSmo chimera consisting of a fusion of the first 633 amino acids (from the N terminus to the end of the seventh TMS) of mouse Smo and the cytoplasmic tail of Drosophila Smo. We show that Cos2 and Fu do co-immunoprecipitate with this chimera (Fig. 6C, lane 7), strongly suggesting that the cytoplasmic tail of Drosophila Smo is necessary to provide interaction with Cos2.

DISCUSSION

Our results show that the human Hh receptors Ptc and Smo are both functionally expressed at the plasma membrane of the Drosophila Schneider 2 cell lines established. Using double-stranded RNA interference directed against Drosophila Ptc, we observed a constitutive activation of the pathway in wild-type S2 cells consistent with previous observations (14). After treatment of S2 cells stably expressing human Ptc with the same dsRNAi, we observed a repression of the pathway in the absence of Hh and the activation of the pathway in the presence of the morphogen. These results demonstrate that human Ptc represses Drosophila Smo activation in the absence of Hh and that Drosophila Hh protein abrogates the repressive effect of human Ptc on Drosophila Smo, resulting in the activation of Hh signal transduction. These observations indicate that human Ptc is able to replace at least partially Drosophila Ptc in the Drosophila Hh pathway. Strikingly, the sequence alignment of human and Drosophila Ptc presented in Fig. 7 shows only 36% identical residues. However, several motifs of 5–15 amino acids are totally conserved between the two species. Two motifs localized in extracellular domains 1 and 2 are present in all Ptc sequences and are very specific to this protein, suggesting that these conserved motifs could be involved in Hh protein interaction. Our results indicate that Drosophila Hh is able to interact with human Ptc and provide support for the involvement of these motifs in Hh interaction. Interestingly, two other motifs localized in extracellular domains 1 and 2 and three in the TMSs 3, 4, 9, and 10 are totally conserved in all Ptc sequences.
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**FIGURE 6. Human Smo does not interact with Costal 2.** A, immunoprecipitation (IP) using Cos2 or HA antibodies and Western blot analysis with anti-Cos2, anti-Fu, anti-dSmo, and anti-HA (for hSmo) from wild-type or stably hSmo-expressing S2 cell extracts treated or not with Hh. hSmo does not co-immunoprecipitate with Cos2 (lanes 5 and 6), and Cos2 does not co-immunoprecipitate with hSmo (lanes 7 and 8). B, hSmo does not interact with the Fu-Cos2 complex in S2 cells treated with dsRNAi-dSmo. Left, Western blot with anti-Fu, anti-Cos2, anti-dSmo, and anti-HA (control for hSmo expression) on cell extracts. Right, immunoprecipitation using dSmo or HA antibodies and Western blot analysis with anti-Cos2, anti-Fu, anti-dSmo, and anti-HA from wild-type or hSmo-expressing S2 extracts treated or not with dsRNAi-dSmo. As a control, Cos2 and Fu co-immunoprecipitate with dSmo (lane 5) but not with the cells treated with dsRNAi-dSmo (lane 6). In the absence of endogenous dSmo, hSmo did not interact with Fu-Cos2 complex (lane 8). C, immunoprecipitation using HA or Myc antibodies with dSmo-HA, hSmo-MAP, or hSmo-dSmo-Myc chimera-expressing cell extracts not treated with Hh. The Western blot analysis was performed with anti-Fu, anti-dSmo, and anti-HA

...that human Smo is expressed in a native conformational state in S2 cells (21), but also in various ABC transporters. A mutation in one of these motifs (G477R in TMS 3) has been shown to abolish Smo repression without compromising the ability of Ptc to bind and endocytose Hh (39). This suggests that, apart from interacting with Hh and endocytosing it, a major and conserved function of Ptc could be the transport of a molecule involved in the inhibition or the activation of Smo (21, 40, 41). The observation that human Ptc is able to inhibit *Drosophila* Smo in the absence of Hh and that Hh binding releases this inhibition suggests that the same molecule is transported by *Drosophila* and human Ptc.

Our results suggest that human Smo is constitutively expressed at the plasma membrane in the S2 cell line selected and is able to bind its antagonist cyclopamine, which suggests that human Smo is expressed in a native conformational state in S2 cells (21). It is now known that there is a remarkable correlation between the localization of Smo and subsequent signaling. In some mammalian cultured cells, Smo is internalized after activation of the pathway instead of accumulating at the cell surface as in *Drosophila*, suggesting that Smo localization might be regulated differently in flies and mammals (17). The hSmo-expressing S2 cell line selected overexpresses the receptor, which is constitutively present at the plasma membrane. We therefore transiently transfected S2 cells with 40 times less hSmo. Both fluorescent anti-hSmo antibody labeling and BODIPY-cyclopamine binding suggest that the presence of Hh increases human Smo at the cell surface. We thus propose that, when expressed in *Drosophila* cells, human Smo is not internalized in response to Hh but is enriched at the plasma cell surface like *Drosophila* Smo. These results are in good agreement with data recently presented by Corbit et al. (19), who showed that mouse Smo becomes localized to cilia in response to Hh signaling. Smo is ∼3-fold enriched in the cilia relative to other parts of the cell in the mouse node, where Hh signaling is active. When Smo was expressed in Madin-Darby canine kidney cells, it was not localized to cilia unless the pathway was activated by treatment with Hh. This signal dependant localization is reminiscent of the situation in *Drosophila*, where Smo activity correlates with its cell surface localization, although, except sensory neurones and spermatids, all other cells in *Drosophila* do not have cilia (42).

Our results suggest that human Smo localization is regulated by Hh but human Smo does not compensate for the absence of Drosophila Smo in dsRNAi-dSmo experiments. We demonstrate that human Smo does not co-immunoprecipitate with Cos2, indicating that human Smo is not able to bind Cos2. In *Drosophila*, in response to Hh, the kinesin Cos2, component of a large cytoplasmic complex composed of at least the serine-threonine kinase Fu and Ci, has been proposed to interact with the cytoplasmic tail of Smo at the plasma membrane and to mediate Smo phosphorylation as well as phosphorylation of the cytoplasmic components Fu and Su(Fu) (14, 15). According to these observations, our experiments performed with the chimeras (for dSmo and hSmo), or anti-Myc (for hSmo-dSmo chimera) antibodies. Cos2 and Fu co-immunoprecipitate with dSmo-HA and hSmo-dSmo-Myc chimera (lanes 6 and 7) but not with hSmo-MAP (lane 5).
мера resulting from the fusion of the N-terminal part of mouse Smo until the end of the seventh TMS and of the cytoplasmic tail of Drosophila Smo indicate that the absence of Cos2 binding with human Smo is due to the lack of a specific Cos2 binding site on the human Smo cytoplasmic tail. This could explain the absence of Cos2 and Fu phosphorylation observed in dsRNAi-dSmo experiments. This is consistent with recent data showing that mouse Smo is insensitive to Drosophila Cos2 in NIH-3T3 cells (38). The sequence alignment of human and Drosophila Smo presented in Fig. 8 shows relatively low homology (only
42%), but, as for Ptc, various motifs are totally conserved essentially in the N-terminal extracellular domain and in the first four TMSs. Structure-function studies of rat Smo suggested that the extracellular domain and the first 2–4 TMSs are necessary for its regulation by Ptc (43, 44). Corbit et al. (19) reported that the localization of Smo to cilia depends on a short motif immediately C-terminal to the last TMS that is present in other G-protein-coupled receptors that localize to cilia. The same motif is present in Drosophila Smo, although Drosophila Hh-responsive cells do not have cilia (see Fig. 8). This motif could be required for membrane localization. Our observations and the high conservation of several motifs between human Smo and Drosophila Smo are in good agreement. They support the proposition of Corbit et al. (19) that this part of the Hh pathway, namely the regulation of Smo activation by Ptc and the Hh-dependent cell surface enrichment of Smo, may be common to both Drosophila cells and ciliated mammalian cells.

The phosphorylation status of Smo appears to modulate its stability at the plasma membrane and its propensity to be further phosphorylated as well as its activity in promoting phosphorylation of Cos2 and Fu (34). The cytoplasmic tail of Drosophila Smo has a cluster of PKA, CKI, and GSK3 consensus phosphorylation sites around Ser-667, -687, and -746 (underlined in Fig. 8). Phosphorylation at these sites is both necessary and sufficient for signal downstream of Smo, since the kinesin Cos2 appears to bind preferentially phosphorylated Smo (32, 34). The corresponding part of human Smo has divergent amino acid sequence, and none of these phosphorylated sites are conserved. Thus, if a comparable phosphorylation-dependent mechanism for activation or stabilization of the Smo active state exists in mammals, it may use distinct kinases and recognition sequences, like the G-protein-coupled receptor kinase-2 (GRK2) (45). Moreover, we can observe on the Smo sequence alignment that human Smo has 230 fewer amino acids than Drosophila Smo at its cytoplasmic tail. The cytoplasmic C-terminal domain of Drosophila Smo has been shown to interact with Cos2 (14), and Zhang et al. (34) proposed that phosphorylation within the Smo cytoplasmic tail indirectly affects Cos2 binding by inducing a conformational shift that promotes interaction of Cos2 with Smo. The difference in this cytoplasmic tail could explain that human Smo is not able to interact with Cos2 and replace Drosophila Smo in the Drosophila Hh pathway. Our observations suggest that the way Smo transduces the Hh signal after activation and cell surface enrichment is different in Drosophila and in humans. The activation mechanism of Smo is still poorly understood in vertebrates.

Taken together, our results and sequence alignment analyses suggest that the part of the Hh pathway involving Hh interaction with Ptc, Hh-dependent cell surface enrichment of Smo, and regulation of Smo by Ptc are highly conserved from Drosophila to humans. In contrast, the mechanism of intracellular Hh signal transduction via activated Smo seems to be different. Consistently, it has been recently shown that Drosophila and mammalian Hh signaling have diverged and that Cos2 and Fu-like activities are absent in mouse animal or cultured cells (38, 46, 47). Finally, the high expression levels of functional human Ptc and human Smo reached in S2 cells could allow the purification of these relevant human membrane proteins. This would allow
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structure-function relationship studies to better understand the Hh signal transduction mechanism and structural studies to develop new therapeutic approaches against tumors and neurodegenerative diseases induced by Hh signaling dysfunction.

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REFERENCES

1. Rubin, G. M., Yandell, M. D., Wortman, J. R., Gabor Miklos, G. L., Nelson, C. R., Hariharan, I. K., Fortini, M. E., Li, P. W., Apweiler, R., Fleischmann, W., Cherry, J. M., Henikoff, S., Skupski, M. P., Misra, S., Ashburner, M., Birney, E., Boguski, M. S., Brody, T., Brokstein, P., Celniker, S. E., Cheritz, S. A., Coates, D., Cravchik, A., Gabrielian, A., Galle, R. F., Gelbart, W. M., George, R. A., Goldstein, L. S., Gong, F., Guan, Y., Harris, N. L., Hay, B. A., Hoskins, R. A., Li, J., Li, Z., Hynes, R. O., Jones, S. J., Kuehl, P. M., Lemate, B., Littleton, J. T., Morrison, D. K., Mungall, C., O’Farrell, P. H., Pickeral, O. K., Shue, C., Vossshall, L. B., Zhang, J., Zhao, Q., Zheng, X. H., and Lewis, S. (2000) Science 280, 2204–2215

2. Ingham, P. W., and McMahon, A. P. (2001) Genes Dev. 15, 3059–3087

3. Hooper, J. E., and Scott, M. P. (1989) Cell 59, 751–765

4. Stone, D. M., Hynes, M., Armanini, M., Swanson, T. A., Gu, Q., Johnson, R. L., Scott, M. P., Pennica, D., Goddard, A., Phillips, H., Noll, M., Hooper, J. E., de Sauvage, F., and Rosenthal, A. (1996) Nature 384, 129–134

5. Therond, P. P., Knight, J. D., Kornberg, T. B., and Bishop, J. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4224–4228

6. Berman, D. M., Karhadkar, S. S., Maitra, A., Montes De Oca, R., Gerstenblith, M. R., Briggs, K., Parker, A. R., Eshleman, J. R., Watkins, J. W., Johnson, G., Gally, S. A., Coates, D., Cravchik, A., Gabrielian, A., Galle, R. F., Gelbart, W. M., George, R. A., Goldstein, L. S., Gong, F., Guan, Y., Harris, N. L., Hay, B. A., Hoskins, R. A., Li, J., Li, Z., Hynes, R. O., Jones, S. J., Kuehl, P. M., Lemate, B., Littleton, J. T., Morrison, D. K., Mungall, C., O’Farrell, P. H., Pickeral, O. K., Shue, C., Vossshall, L. B., Zhang, J., Zhao, Q., Zheng, X. H., and Lewis, S. (2000) Science 280, 2204–2215

7. Thayer, S. P., di Magliano, M. P., Heiser, P. W., Nielsen, C. M., Roberts, D. I., Lawers, G. Y., Qi, Y. P., Gysin, S., Fernandez-del Castillo, C., Yajnik, V., Antoniu, B., McMahon, M., Warshaw, A. L., and Hebrok, M. (2003) Nature 422, 313–317

8. Watkins, D. N., Berman, D. M., Burkholder, S. G., Wang, B., Beachy, P. A., and Baylin, S. B. (2003) Nature 422, 313–317

9. Reya, T., Morrison, S. J., Clarke, M. F., and Weissman, I. L. (2001) Nature 414, 105–111

10. Ruiz i Altaba, A., Sanchez, P., and Dahmane, N. (2002) Nat. Rev. Cancer 2, 361–372

11. Lai, K., Kaspar, B. K., Gage, F. H., and Schaffer, D. V. (2003) Nat. Neurosci. 6, 21–27

12. Marigo, V., Davey, R. A., Zuo, Y., Cunningham, J. I., and Tabin, C. J. (1996) Nature 384, 176–179

13. Torroja, C., Gorfrinkiel, N., and Guerrero, I. (2005) J. Neurobiol. 64, 334–356

14. Ruel, L., Rodriguez, R., Gallet, A., Lavenant-Staccini, L., and Therond, P. P. (2003) Nat. Cell Biol. 5, 907–913

15. Lum, L., Yao, S., Mazer, B., Rovescalli, A., Von Kessler, D., Nirenberg, M., and Beachy, P. A. (2003) Science 299, 2039–2045

16. Ogden, S. K., Ascano, M., Jr., Stiegman, M. A., Suber, L. M., Hooper, J. E., and Robbins, D. J. (2003) Curr. Biol. 13, 1998–2003

17. Incardona, J. P., Gruenberg, J., and Roelink, H. (2002) Curr. Biol. 12, 983–995

18. Chen, W., Ren, X. R., Nelson, C. D., Barak, L. S., Chen, J. K., Beachy, P. A., de Sauvage, F., and Letkowitz, R. J. (2004) Science 306, 2257–2260