The Drosophila *smoothened* Gene Encodes a Seven-Pass Membrane Protein, a Putative Receptor for the Hedgehog Signal

Joy Alcedo,* Marina Ayzenzon,† Tonia Von Ohlen,†
Markus Noll,* and Joan E. Hooper†

*Institut für Molekularbiologie II der Universität Zürich
CH-8057 Zürich
Switzerland
†Department of Cellular and Structural Biology
University of Colorado Health Sciences Center
Denver, Colorado 80262

Summary

*smoothened* (smo) is a segment polarity gene required for correct patterning of every segment in Drosophila. The earliest defect in smo mutant embryos is loss of expression of the Hedgehog-responsive gene wingless between 1 and 2 hr after gastrulation. Since smo mutant embryos cannot respond to exogenous Hedgehog (Hh) but can respond to exogenous Wingless, the smo product functions in Hh signaling. Smo acts downstream of or in parallel to Patched, an antagonist of the Hh signal. The smo gene encodes an integral membrane protein with characteristics of G protein-coupled receptors and shows homology to the Drosophila Frizzled protein. Based on its predicted physical characteristics and on its position in the Hh signaling pathway, we suggest that smo encodes a receptor for the Hh signal.

Introduction

The development of a multicellular organism depends on mechanisms that initially specify and subsequently maintain positional information. In Drosophila embryos, a cascade of transcription factors progressively defines position along the antero-posterior axis with increasing precision before cellular blastoderm (Nüsslein-Volhard and Wieschaus, 1980; reviewed by Small and Levine, 1991; St Johnston and Nüsslein-Volhard, 1992). At cellular blastoderm and later stages of development, information is maintained across cell borders by signal transduction pathways, the components of which are encoded by segment polarity genes (reviewed by Peifer and Bejsovec, 1992). Two such pathways are initiated by the extracellular signals Wingless (Wg) and Hedgehog (Hh). These secreted proteins (van den Heuvel et al., 1989; González et al., 1991; Lee et al., 1992; Taylor et al., 1993; Tabata and Kornberg, 1994) are synthesized in adjacent stripes of cells to delineate and maintain the parasegmental boundaries, which initially define the metameric pattern in the larva and the adult (Martinez-Arias and Lawrence, 1985). Expression of Wg and Hh are tightly linked during gastrulation and germ band extension. During these stages, Wg maintains, through a series of partially known steps, expression of the homeodomain transcription factor Engrailed (En; Siegfried et al., 1992, 1994), which represses the *cubitus interruptus* (*ci*) gene (Eaton and Kornberg, 1990) encoding a zinc-finger transcription factor (Orenic et al., 1990). Since the Ci protein represses hh, repression of *ci* by En activates *hh* transcription (Domínguez et al., 1996). The secreted Hh protein in turn triggers a signal transduction cascade that activates Wg (DiNardo et al., 1988; Hidalgo and Ingham, 1990; Ingham, 1993; Ingham and Hidalgo, 1993) and at least another segment polarity gene, patched (*ptc*; Hidalgo and Ingham, 1990; Tabata and Kornberg, 1994). Transcription of Wg is further stimulated in an autocrine loop by its own protein product (Hooper, 1994; Yoffe et al., 1995). The short range of both signals dictates that Wg and Hh expression patterns are restricted to adjacent narrow stripes of cells (Vincent and Lawrence, 1994; Porter et al., 1995). Anything that interferes with either signal quickly leads to loss of the other signal and to catastrophic failure of segmentation. By the extended germ band stage, expression of Wg and Hh is no longer linked since *en* becomes autoregulatory (Bejsovec and Martinez Arias, 1991; Heemskerk et al., 1991), and Wg is maintained by an autocrine loop (Li and Noll, 1993). As development proceeds, Hh and Wg signals act as morphogens to specify many aspects of the larval cuticle pattern (Bejsovec and Wieschaus, 1993; Heemskerk and DiNardo, 1994). Although some of the functions of these signals in morphogenesis are known, the receptors required to transduce them have remained elusive. Candidates for the receptors might be among the segment polarity genes that have not yet been isolated and whose phenotypes resemble that of *hh* or *wg*. One of the segment polarity genes that fulfills this criterion is *smoothened* (*smo*). Another that has been postulated to encode the Hh receptor is *ptc* (Ingham et al., 1991). The activity of the Ptc product, which is a multiple membrane-spanning cell-surface protein (Hooper and Scott, 1989; Nakano et al., 1989), represses the *wg* gene and is antagonized by the Hh signal (Ingham et al., 1991). However, it cannot be the only Hh receptor, since Hh has effects in *ptc* null embryos (Bejsovec and Wieschaus, 1993). Other segment polarity gene products implicated in the Hh signal transduction pathway but clearly not functioning as Hh receptor are the zinfc-finger protein Ci and two serine/threonine protein kinases, Fused (Fu) and cyclic AMP–dependent protein kinase A (PKA; reviewed by Perrimon, 1995).

This study describes a genetic and molecular characterization of the smo gene. We show that *smo* is necessary for Hh signaling and that it acts downstream of *hh* and *ptc*. It encodes a protein with many structural features of the serpentine family of heterotrimeric G protein-coupled receptors and is homologous to the Drosophila frizzled gene. Based on genetic and molecular analyses, we propose that Smo is the receptor of the Hh signal.

Results

The *smoothened* Segment Polarity Phenotype

The *smo* gene was identified as a segment polarity gene and initially named smooth (Nüsslein-Volhard et al.,
1984). Since this name already described another locus, the new segment polarity gene was renamed smoothened (Lindsley and Zimm, 1992). Nüsslein-Volhard et al. (1984) recovered three recessive zygotic lethal alleles of smo, which are all cold-sensitive. At 25°C, the morphological defects are mild, while at 18°C, smo embryos exhibit a classic segment polarity cuticle phenotype (Nüsslein-Volhard et al., 1984). There is variability in the severity of the cuticle phenotype. Some individuals have lost most segmental modulation apparent in the wild type and resemble hh or wg null embryos (Jürgens et al., 1984; Nüsslein-Volhard et al., 1984). Others retain considerable segmental modulation of denticle type and polarity (data not shown).

The variability and cold sensitivity suggest that these embryos retain residual smo function, possibly because the alleles are not null or because of a maternal effect. It is difficult to assess the smo null phenotype, since the available deficiencies that uncover smo do not survive beyond early embryonic stages. However, smo1/Df and smo2/Df exhibit a range and frequency of phenotypes similar to homozygotes and transheterozygotes of all three smo alleles, suggesting that these behave as near null alleles at 18°C. Although we have not tested whether a maternal contribution of smo− to the developing oo-
cyte accounts for the phenotypic variability of homozy-
gous or hemizygous smo embryos, the considerable
levels of smo mRNA in 0–2 hr embryos revealed by
Northern blots (data not shown) renders this explanation plausible. The remaining analysis presented here reports phenotypes seen in the majority of smo mutant embryos raised at 18°C.

A classic segment polarity cuticular phenotype, such as that of smo mutant embryos, predicts loss of Wg and Hh signaling at the parasegment border in the ectoderm before stage 11 (reviewed by Perrimon, 1994). After their initial activation by pair-rule gene products in neighboring stripes of epidermal cells, Wg and Hh expression become interdependent. In the absence of Wg signaling, e.g., in wg−/− embryos, loss of En protein slightly precedes that of Wg during stage 9 and early stage 10 (Figures 1A and 1B; DiNardo et al., 1988; Martinez Arias et al., 1988; Bejsövec and Martinez Arias, 1991; Heemskerk et al., 1991; Hidalgo, 1991; Peifer et al., 1991; Ingham and Hidalgo, 1993), because Wg signaling directly stimulates the expression of both genes (Bejsövec and Martinez Arias, 1991; Hooper, 1994; Yoffe et al., 1995). In the absence of Hh signaling, e.g., in hh−/− embryos, Wg protein is lost during stage 9 (Hidalgo and Ingham, 1990; Hidalgo, 1991; Ingham and Hidalgo, 1993), followed by loss of En ventrally and dorsally during late stage 9 and early stage 10 (DiNardo et al., 1988). Thus, in hh−/− embryos, Wg protein has nearly completely decayed by late stage 9, when En protein only begins to disappear (Figure 1C). In smo mutant embryos, Wg and En expression are initiated normally at the cellular blastoderm stage (data not shown). Wg protein subsequently decays during stage 9 (van den Heuvel et al., 1993), clearly before En protein begins to decay (Figure 1D). Therefore, the temporal loss of En protein in smo embryos resembles that in hh embryos and is different from that in wg embryos, consistent with a role for Smo in Hh rather than paracrine Wg signaling.

Figure 1. The smoothened Phenotype Resembles the hedgehog Rather Than the wingless Phenotype
Lateral epidermal expression patterns of the Wg protein (black) and the En protein (orange) are shown for wild-type (A), wg1118 (B), hh108 (C), and smo− (D) embryos at late stage 9 (B–D) or early stage 10 (A) (Campos-Ortega and Hartenstein, 1985). En decays earlier in wg embryos than in hh and smo embryos.

smoothened Acts in Hh or Autocrine Wg Signal Transduction
To assess the requirement for smo− function in Wg versus Hh signaling, we have expressed each of these signals ectopically, under the control of heterologous promoters in smo null mutant embryos. When Wg is expressed ectopically under the control of the pair-rule hairy promoter in alternate parasegments of hGAL4 UASwg embryos, it drives ectopic expression of En in odd-numbered stripes that, as compared to wild-type En stripes (Figure 2A), are expanded posteriorly (Figure 2B; Yoffe et al., 1995), even in the absence of a functional Smo protein (Figure 2C). Thus, smo− is not essential for this response to paracrine Wg signaling. Even-numbered En stripes are unaffected by the ectopic Wg signal, as expected (Figures 2B and 2C). In smo embryos, all En bands eventually decay (Figures 2C and 2D).

When Hh is ubiquitously expressed under the control
**Figure 2. Ectopic Expression of Wg Activates Ectopic En Expression in Both Wild-Type and smo Embryos**

En protein expression is shown in wild-type (A), hGAL4 UASwg (B), smo/− smo/−; hGAL4 UASwg (C), and smo/− smo/− embryos (D). Compared to wild-type embryos (A), odd-numbered En stripes are expanded posteriorly in hGAL4 UASwg embryos (B–C) because of ectopic Wg expression in odd-numbered parasegments (Yoffe et al., 1995). In smo/− smo/−; hGAL4 UASwg embryos (C), even parasegments show the weak En expression characteristic of smo mutants (D). Thus, Wg protein can induce a response in smo mutant embryos. Stage 11 embryos were cut along the amnioserosa, unfolded, and flattened to show En expression in the epidermis of parasegments 5–14.

**Figure 3. Ubiquitous Hh Expression Cannot Activate Wg Expression in smo Embryos**

Wg expression patterns of wild-type (A), heat-shocked Hshh (B), and smo/− smo/− whole-mount embryos (C) are shown at stage 11. The expected ratios of the three different phenotypes (wild-type narrow Wg stripes, Hshh–induced broad Wg stripes, and decay of Wg protein characteristic of smo embryos) among the progeny of a cross between smo/− CyO and smo/− ; Hshh/ + flies are listed in (D) for smo being in either the Hh or Wg signaling pathway. Statistical analysis of the number (N) and relative ratios (including standard deviations) of observed Wg expression phenotypes indicate that Smo is in the Hh rather than in the Wg signaling pathway.

<table>
<thead>
<tr>
<th>phenotypes of progeny</th>
<th>expected % of embryos if smo is in Hh or Wg pathway</th>
<th>observed embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wild type</td>
<td>smo/− smo/−</td>
</tr>
<tr>
<td></td>
<td>37.5</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>37.5</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>N = 92</td>
<td>N = 49</td>
</tr>
<tr>
<td></td>
<td>% = 41 ± 5</td>
<td>% = 22 ± 5</td>
</tr>
</tbody>
</table>
We shall refer to this genetically defined interval as the smo region.

The smo gene was cloned by extending a chromosomal walk, comprising the deficiency Df(2L)al at 21C (Schneitz et al., 1993), toward the left telomere to include the breakpoint of the deficiency Df(2L)PMF and thus the smo region (Figure 5). The breakpoint of Df(2L)PMF was mapped to a region between –294 and –295 on the chromosomal walk, while the distal breakpoint of Df(2L)al is located between –257 and –260 (Schneitz et al., 1993). Thus, the genetically defined smo region, which must contain all sequences essential for smo function, lies between coordinates –257 and –295 (Figure 5).

To locate transcripts within this region that appear at the time when smo function is required, Northern blots of poly(A) RNA from 4–8 hr old embryos were screened by hybridization with probes from across the smo region. At least six different transcripts were identified in 4–8 hr embryonic mRNAs within this 38 kb interval. To pinpoint the smo transcript, five overlapping genomic DNA fragments were introduced into the germline by P element-mediated transformation. When crossed into a smo background, only one of these, a 12.1 kb AatII fragment (Figure 5), complemented smo. More detailed mapping of isolated cDNAs corresponding to the transcripts of this genomic fragment revealed that it contained three independent transcription units. While a 9.1 kb AatII–Apal fragment harboring the two distal transcription units failed to complement smo, a 6.2 kb HindIII–AatII fragment, which encodes a 4.2 kb mRNA, the 5′ end of a flanking distal transcript, and the 3′ end of a flanking proximal transcript, was found to contain all smo rescuing activity (Figure 5). One copy of this fragment fully rescued all aspects of the smo phenotype and hence includes all essential sequences for expression and function of the smo gene. The encoded 4.2 kb mRNA is the smo transcript.

Genomic and cDNA sequencing of the smo transcription unit reveals six exons spread over 4584 bp (Figure 5). They generate an mRNA whose length is 4005 nt without its poly(A) tail. The transcription start site was identified by two independent clones using the 5′ rapid
smoothened Encodes a Putative Hedgehog Receptor

Figure 5. Cloning and Identification of the smo Gene

(Top) The proximal breakpoint of Df(2L)PMF and the distal breakpoint of Df(2L)al are indicated with respect to a genomic EcoRI map (scale in kb; broken lines are EcoRI sites of neighboring fragments whose order has not been determined), derived from a chromosomal walk of the region between 21B7–8 and 21C1 (Y12–3, Y11–7, Y10–3, and BPal-1 are inserts isolated from a genomic library prepared in EMBL4), a distal extension (H.-P. Lerch and M. N., unpublished data) of a walk covering the Df(2L)al (Schneitz et al., 1993). Stippled boxes delimit the EcoRI fragments that include the deficiency breakpoints.

(Middle) An enlarged map of clone Y11–7 is shown, below which the orientation and extent of the smo transcript and three unknown transcripts are indicated by arrows (dashed line is uncertainty of 5' end of the most proximal transcript). Underneath, the genomic inserts of three P-element constructs that rescue (P[AatII;w1] and P[HindIII-AatII;w1]) or fail to rescue (P[AatII-ApaI;w1]) smo mutants are shown.

(Bottom) A restriction map of an enlarged genomic fragment included in the P-element rescue construct P[HindIII-AatII, w1] is shown with the intron/exon structure and the open reading frame (in black) of the smo gene. (Aa) AatII; (Ap) ApaI; (B) BamHI; (Bg) BglII; (Bs) BspEI; (C) ClaI; (H) HindIII; (Pv) PvuII; (R) EcoRI; (RV) EcoRV; (S) Sait; (X) Xhol.

amplification of cDNA ends (RACE) technique. A possible TATA box lies 18 bp upstream of the transcriptional start site, and the canonical polyadenylation signal AATAAA is found 17 nt upstream of the poly(A) tract.

Northern blots using staged mRNAs and in situ hybridizations to embryos and imaginal discs show that the smo gene is expressed at all developmental stages, though the levels vary (data not shown). The polyadenylated transcript migrates as a single species of 4.2 kb, relative to DNA size markers. This is consistent with the 4005 bp of the full-length cDNA measured by sequencing.

smo Encodes a Putative G Protein–Coupled Receptor

Conceptual translation of the smo cDNA reveals a single large open reading frame encoding a protein of 1036 amino acids with four methionines among its first 14 residues (Figure 6). While the sequence preceding the second ATG of the open reading frame fits best to the Kozak consensus for vertebrate initiation (Kozak, 1987), the sequence preceding the third ATG is a better match to the consensus for initiation of translation in Drosophila (Cavener, 1987). However, none of the four potential translation initiation sites shows a high homology to either consensus sequence. Using the second or third ATG as initiator codon, the predicted protein products consist of 1028 or 1024 amino acids with molecular masses of 115.5 or 115.0 kDa. Hydropathy analysis predicts that the putative Smo protein is an integral membrane protein with seven membrane spanning α helices and a long hydrophilic C-terminal tail (Figure 6). An additional hydrophobic segment near the N-terminus has characteristics of a signal peptide (von Heijne, 1986). Its cleavage would generate an N-terminal extracellular domain that includes five potential N-linked glycosylation sites (Figure 6). Two additional N-linked glycosylation sites are found in the putative first and second extracellular loops between the transmembrane α helices. These structural predictions suggest that Smo belongs to the serpentine receptor family, whose members are all coupled to G proteins (reviewed by Kobilka, 1992; Strader et al., 1994).

While N-terminal extracellular domains of the size found in Smo (226 amino acids after cleavage of the putative signal peptide) are common among G protein–coupled receptors whose ligands are peptides or glycoproteins rather than biogenic amines, the large C-terminal domain of Smo (481 amino acids) is unusual. Nevertheless, it includes five potential phosphorylation sites for PKA that, together with the PKA site of the second intracellular loop, might serve desensitization of
Figure 6. Deduced Amino Acid Sequence of the Smo Protein

The putative amino acid sequence of the Smo protein, derived from cDNA, 5’ RACE, and genomic DNA sequences, is shown. The predicted signal peptide sequence (von Heijne, 1986) and seven transmembrane domains (TMpred program, based on the statistical analysis of the TMbase database [Hofmann and Stoffel, 1993]) are underlined. Seven potential N-linked glycosylation sites are boxed (Gavel and von Heijne, 1990), while six putative PKA phosphorylation sites are circled (Glass et al., 1986). The positions at which the coding region is interrupted by five introns are indicated by triangles. Amino acids are numbered in the left margin.

Smo by its uncoupling from the Gα protein subunit as structural similarities between the extracellular portions of Smo and Fz (Strader et al., 1994). has been observed for serpentine receptors (Kobilka, 1992).

Discussion

The smo gene is required for the maintenance of segmentation in Drosophila embryos (Nüsslein-Volhard et al., 1984). This maintenance depends crucially on segmentally repeated Hh and Wg signaling at the parasegmental boundaries. The secreted Hh and Wg protein signals maintain each other’s synthesis in neighboring stripes and thereby establish signaling centers, which organize the segmental pattern of the epidermis (Bejsovec and Wieschaus, 1993; Heemskerk and DiNardo, 1994; reviewed by Perrimon, 1994). The lack of segmentation in smo mutant embryos might therefore be explained if either Wg or Hh signaling or both depended on smo activity. We have shown here that in Wg mutant embryos, En decays during stage 9, while in h and smo expression (Eaton and Kornberg, 1990; Domínguez et al., 1996), is not dependent on smo.
Figure 7. Homology Between Smo and Fz Proteins

The predicted amino acid sequences of the N-terminal portion of the Smo protein and the entire Fz protein from Drosophila melanogaster (Vinson et al., 1989) were compared using the Wisconsin Package, Version 8.0 (Genetics Computer Group, 1994) and the BOXSHADE 3.0 program (Hofmann and Baron, Bioinformatics Group, Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland). Identity of amino acids are shaded in black, similarity in gray. The seven conserved transmembrane domains, predicted by using the TMpred program on either sequence (Hofmann and Stoffel, 1993), are indicated as TM1-TM7.

Instead, smo is required for maintenance of wg expression during stages 9 and 10, a process that depends on both the Hh signal and an autocrine Wg signal (Hooper, 1994; Yoffe et al., 1995). A pair of additional observations rule out a role of smo in paracrine Wg signaling but are consistent with a role in Hh or autocrine Wg signaling. In smo mutant embryos, exogenous Wg maintains En expression beyond stage 10 (Figure 2), and exogenous Hh expression is unable to rescue Wg expression (Figure 3).

samo May Encode the Receptor for the Hh Signal

The amino acid sequence encoded by smo establishes that Smo belongs to the Fz family of seven-pass membrane receptors (Figures 6 and 7). Smo could be the Hh receptor or the autocrine, but not paracrine, Wg receptor. If Smo is the autocrine Wg receptor, then an additional Wg receptor would have to transmit the paracrine signal. However, in all studied cases of autocrine and paracrine signaling, signal molecules use the same receptors in both modes. Moreover, in known cases where the paracrine and autocrine pathways differ, they always diverge downstream of their common receptors.

Our recent unpublished results are also inconsistent with the proposal that Smo is an autocrine Wg receptor. Specifically, ectopic expression of Wg activates gsb in an autocrine fashion (Li and Noll, 1993) even in the absence of smo (data not shown). This activation of gsb by Wg does not depend on smo and hence excludes smo from being in the autocrine Wg signaling pathway. We conclude that Smo is involved neither in paracrine nor autocrine Wg signaling but acts downstream of the Hh signal. Moreover, the finding that smo encodes a G protein-coupled receptor is in excellent agreement with the observed role of PKA in Hh signal transduction (Jiang and Struhl, 1995; Johnson et al., 1995; Lepage et al., 1994; Yoffe et al., 1995). Taken together, all of these observations and considerations argue that Smo is the receptor for the Hh signal.

Ptc was previously proposed to be a constitutively active receptor that was inactivated by binding of Hh, thus permitting transcription of Hh-target genes (Ingham et al., 1991). Specifically, ptc is a negative regulator of expression of the Hh-target genes wg, ptc, and dpp and is epistatic to hh with respect to these markers (Martinez Arias et al., 1988; Hidalgo and Ingham, 1990; Ingham et al., 1991; Hidalgo, 1991; Sampedro and Guerrero, 1991; Ingham, 1993; Basler and Struhl, 1994; Capdevila et al., 1994; Schuske et al., 1994; Tabata and Kornberg, 1994). However, in the absence of Ptc, the Hh signal still enhances wg transcription (Bejsovec and Wieschaus, 1993). It follows that ptc is not strictly epistatic to hh and thus eliminates the necessity to postulate that Ptc acts as a receptor for the Hh signal. We propose a different role for Ptc: it serves as an accessory protein that might be associated with Smo and regulates its sensitivity to the Hh signal (Figure 8).

A Model for the Transduction of the Hh Signal

The transduction of the Hh signal in the epidermis of embryos and in imaginal discs is very similar (reviewed by Ingham, 1995). Ptc antagonizes the activation by the Hh signal of the target genes wg and ptc (Ingham et al., 1991; Hidalgo and Ingham, 1990; Ingham et al., 1991; Hidalgo, 1991; Sampedro and Guerrero, 1991; Ingham, 1993; Basler and Struhl, 1994; Capdevila et al., 1994; Schuske et al., 1994; Tabata and Kornberg, 1994). However, in the absence of Ptc, the Hh signal still enhances wg transcription (Bejsovec and Wieschaus, 1993). It follows that ptc is not strictly epistatic to hh and thus eliminates the necessity to postulate that Ptc acts as a receptor for the Hh signal. We propose a different role for Ptc: it serves as an accessory protein that might be associated with Smo and regulates its sensitivity to the Hh signal (Figure 8).
Another factor known to regulate Hh-target genes is PKA (Jiang and Struhl, 1995; Johnson et al., 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995; Strutt et al., 1995). Similar to Ptc, PKA inhibits Ci activity as evident from the fact that inhibition or loss of PKA function activates wg and ptc in the absence of an Hh signal (Jiang and Struhl, 1995; Li et al., 1995; Lepage et al., 1995). Thus, PKA is epistatic to Hh and hence acts downstream or in parallel to Hh signaling (Jiang and Struhl, 1995; Li et al., 1995). Moreover, as Ptc is unable to inhibit transcription of its own gene in pka− clones, PKA is also epistatic to Ptc and thus acts downstream or in parallel to Ptc (Jiang and Struhl, 1995; Li et al., 1995). Neither Hh nor Ptc acts exclusively through PKA, as attested by the following observations. A constitutive level of PKA activity, one that is sufficient to suppress ectopic activation of ptc in pka− clones, is unable to counteract the effect of the Hh signal at the antero-posterior border (Li et al., 1995) and does not prevent the ectopic expression of Hh-target genes in ptc− clones (Jiang and Struhl, 1995; Li et al., 1995). That PKA is not the only effector of the Hh signal is also consistent with the observation that loss of PKA activity does not produce all consequences of Hh signaling (Jiang and Struhl, 1995).

These findings, combined with the results reported here, are explained best by the model illustrated in Figure 8. We propose that smo encodes a G protein-coupled receptor that responds to the extracellular Hh signal via a trimeric G protein inhibiting adenylate cyclase (Figure 8). Ptc and Hh act in parallel on Fu and Ci to regulate transcription of wg and ptc. Ptc and PKA inhibit activation of wg and ptc by Ci, while Hh counteracts this inhibition. This is consistent with the observation that Ptc does not act exclusively through PKA (Jiang and Struhl, 1995; Li et al., 1995) but also acts in a parallel pathway.

An important difference between the inhibitory activities of Ptc and PKA is that loss of PKA function activates Hh-target genes in the absence of a functional Hh signal regardless of Ptc concentrations, whereas loss of Ptc function is unable to activate Hh-target genes at high levels of constitutive PKA activity (Li et al., 1995). These findings imply, first, that the ability of Ptc to inhibit activation of Hh-target genes depends on PKA and second, that PKA does not act on Ptc but on a component downstream of Ptc (Figure 8). Recent results show that high PKA activity cannot counteract the phosphorylation of Fu that depends on the Hh signal but can be inhibited by Ptc, suggesting that Fu indeed acts downstream of Hh and Ptc (Théron et al., 1996). It further follows that PKA does not act on a component between Ptc and Fu. It is unclear, however, whether PKA acts through Fu or a component downstream of Fu. It has been assumed that Hh signaling depends entirely on Fu because maintenance of wg expression depends on Fu activity already during stage 9 (Forbes et al., 1993) and because fu is epistatic to ectopic Hs-hh expression (Ingham, 1993). However, since the early maintenance of wg expression depends not only on Hh but also on an autocrine Wg signal (Hooper, 1994; Yoffe et al., 1995), Fu may be required in the autocrine Wg signaling pathway. In this case, the PKA-dependent transduction of the Hh signal...
does not have to act through Fu but could converge with the Ptc-dependent pathway at a component below Fu, for example on Costal-2 (Prät et al., 1993) or Ci. Regardless of whether or not PKA acts through Fu, the activation of ptc by Ci is determined by the relative activities of PKA and Ptc.

The antagonistic effects exerted by Hh and Ptc are further evident from the observation that both loss of ptc function and ectopic Hh expression result in ectopic wg activation (Ingham et al., 1991; Ingham, 1993; Tabata and Kornberg, 1994). Moreover, in the absence of both antagonists Hh and Ptc, the Hh signaling pathway is constitutively active because wg remains expressed in ptc hh double mutants (Ingham et al., 1991). We now find that this activity completely depends on Smo, even in the presence of Hh, since wg expression is no longer maintained in smo ptc double mutants (Figure 4). It follows that Smo is constitutively active in ptc hh embryos. This constitutive activity of Smo is stimulated by the Hh signal (Bejsovec and Wieschaus, 1993) and inhibited by Ptc in the absence of Hh. Because the effects of Ptc and Hh are parallel and linked, as argued above, Hh overrides the inhibition by Ptc by counteracting the inhibitory effects of both PKA and Ptc on Fu and Ci (Figure 8). Ptc might inhibit constitutive Smo signaling by acting through Fu (Thérond et al., 1996). On the other hand, Hh overcomes this inhibition as well as that of PKA.

The simplest possible mechanism by which Hh relieves the inhibition by Ptc takes into consideration the fact that both Smo and Ptc (Hooper and Scott, 1989; Nakano et al., 1989) are integral membrane proteins and assumes that Ptc is inactivated by its association with the Hh–Smo complex. It is also possible that Ptc in its active form is already associated with Smo in the absence of bound Hh (Figure 8). In that case, when Hh binds to the Smo–Ptc complex, Ptc is released from its association with Smo, thus abolishing its inhibitory activity on Fu (Thérond et al., 1996). As a further consequence, PKA is inhibited by G protein–coupled signaling (Figure 8). Alternatively, upon binding to the Smo receptor, Hh might counteract the inhibition of Ptc by generating two active signaling moieties, the Gαi and the Gβγ subunits that generally have higher and lower affinities, respectively, for their effectors. Either signaling moiety may inhibit the action of Ptc on Fu. Different affinities of Gαi and Gβγ for their respective effectors would provide a simple explanation for qualitatively different responses to low and high levels of Hh signaling (Heemskerk and DiNardo, 1994).

**General Features and Implications of the Model**

Several features of our model are new and noteworthy. Smo has constitutive signaling activity (Figure 4) that has been observed only for mutated G protein–coupled receptors (reviewed by Strader et al., 1994). This constitutive activity is inhibited and the sensitivity of Smo for the Hh signal is reduced by the Ptc protein, which plays the role of an accessory protein that modulates the activity of the Smo receptor. Ptc thus restricts the range of the Hh signal by enhancing its threshold concentration for signal transduction through Smo. An analogous situation might exist for the transduction of the Wg signal, in which Nkd appears to restrict the range of the Wg signal by increasing its threshold concentration (Siegfried et al., 1992). Both properties of Smo that are unusual for a receptor coupled to a trimeric G protein, namely its constitutive activity and its inhibition by the accessory protein Ptc, might require additional cytoplasmic domains, which would explain the unusually large cytoplasmic C-terminal moiety of Smo. The balance between the antagonistic Hh signal and Ptc affects at least two parallel pathways (Figure 8). Hh mediates its effect by inhibiting PKA as well as by counteracting the Ptc-dependent inhibition of Fu and possibly of Smo. Thus, Hh acts by antagonizing both PKA and Ptc. Like Hh, Ptc may also play two roles (Figure 8). It inhibits the constitutive signaling of the Smo receptor by direct association and prevents signaling through Fu and Ci.

What would be the advantage of such a complex signaling network? A possible clue might be provided by considering that Ptc on the one hand represses its own activation and on the other hand reduces the sensitivity of the Smo receptor for the Hh signal, and that both Smo and Ptc (Hooper and Scott, 1989; Nakano et al., 1989) are integral membrane proteins and assumes that Ptc is inactivated by its association with the Hh–Smo complex. It is also possible that Ptc in its active form is already associated with Smo in the absence of bound Hh (Figure 8). In that case, when Hh binds to the Smo–Ptc complex, Ptc is released from its association with Smo, thus abolishing its inhibitory activity on Fu (Thérond et al., 1996). As a further consequence, PKA is inhibited by G protein–coupled signaling (Figure 8). Alternatively, upon binding to the Smo receptor, Hh might counteract the inhibition of Ptc by generating two active signaling moieties, the Gαi and the Gβγ subunits that generally have higher and lower affinities, respectively, for their effectors. Either signaling moiety may inhibit the action of Ptc on Fu. Different affinities of Gαi and Gβγ for their respective effectors would provide a simple explanation for qualitatively different responses to low and high levels of Hh signaling (Heemskerk and DiNardo, 1994).

**Experimental Procedures**

_Drosophila Stocks and Genetics_

Three EMS–induced alleles, smo¹, smo², and smo³, were obtained from the Tubingen stock center (Nüsslein-Volhard et al., 1984). Both
the deficiency Di(2L) pda 8 Tg [21B; 21C6], derived as recombinant from In(2L) pda [21B; 22F1–2] and In(2L) Tg [21C6; 22F1–2], and the terminal duplication Dp[2L] fushii/7 [21A; 21C6], which covers a haplo-
lethality of the deficiency, were generated and kindly provided as Dp[2L] pda 8 Tg [21B; 21C6] and Dp[2L] fushii/7 [21A; 21C6], for the genotype, in the stock by Pascal Heitzler
(IGSMC, Strasbourg, France), smo/Df refers to results obtained with both Di(2L) pda 8 Tg a and Di(2L) fushii/7. The w; H GAL4[w+] UAS lwg[w+] /TM3, Sb stock (Wieland, Philadelphia, PA), while w; H-hw[w+]/
TM3, Ser flies (Ingham, 1993) were obtained from Phil Ingham (London, UK). The ptc apllele, an enhancer trap line with Flac2J inserted into the 3′ end of ptc, was provided by Corey Goodman (Berke-
ly, CA). All other stocks were obtained from the Bloomington Stock Center and are described by Lindsley and Zimm (1992).

All Drosophila culture and genetics utilized standard techniques except that all embryos were collected and aged at 18°C to maximize expression of the smo phenotype, with the exception of the wg+ alleles embryos that were collected at 25°C. Embryos were staged by mor-
phology according to Campos-Ortega and Hartenstein (1985). Chro-
mosomes scored as smo in mapping crosses were tested against at least two different smo alleles for viability and for the smo cuticle phenotype.

The effect of ectopic Wg expression in smo mutant embryos was assayed by crossing smo cn bw sp/CyO virgins with smo b pr/+ ;
H GAL4 UAS lwg/+ males. Progeny were collected for 12–16 h at
18°C, fixed, and assayed for En protein.

The effect of ectopic Hh expression in smo embryos was assayed by crossing smo cn bw sp/CyO virgins with smo b pr/++;
h GAL4 UAS lwg/++; males. Progeny were collected for 12–16 h at
18°C, fixed, and assayed for En protein.

The smo ptc phenotype was determined by collecting embryos from smo b pr Df(2R)44CE bw sp/CyO and from smo ptc P[w+;
lacW] bw sp/CyO parents at 18°C. The embryos were fixed and assayed for both Wg RNA, Galp protein, and En protein. Similar results were obtained for crosses among each genotype, where approximately 75% of the offspring showed wild-type expression patterns and the remaining embryos displayed the distinct mutant pattern.

Genomic rescue experiments used the P-element vector pCASpeR4 (Pirrotta, 1988), Df(1)w67c2, y host flies, and standard techniques (Rubin and Spradling, 1982). Rescuing activity was de-
efined by restoration of full viability, fertility, and normal morphology to smo/ smo flies.

Immunohistochemistry and In Situ Hybridization
Detection of Wg protein was performed by staining embryos with rabbit anti-Wg antisera (van den Heuvel et al., 1993; gift of Roel Nuesse, Stanford University). En immunohistochemistry used the 4D1 monoclonal antibody (gift of Tom Kornberg), while Gsb immunohis-
tochemistry used a 1:1 mix of two monoclonals, 10E10 and 16F12 (Zhang et al., 1994; gift of Robert Holmgren, Northwestern Uni-
versity). The primary antibodies were detected with the Vectastain ABC detection system (Vector Laboratories, Burlingame, CA) or with an alkaline phosphatase conjugate of affinity-purified goat anti-mouse IgG H + L (Jackson ImmunoResearch Laboratories, West Grove, PA) according to standard procedures (Kania et al., 1990). Double-label-
ing of Wg and En proteins was carried out according to Lawrence et al. (1987), with a slight modification. To visualize En as the second label, aminothiazole carbazol (Sigma Chemical Co., St. Louis, MO) was used as a substrate for horseradish peroxidase, which yields an orange product in the presence of hydrogen peroxide (Harlow and Lane, 1988).

In situ hybridizations utilized antisense-strand riboprobes and were performed according to standard procedures (Tautz and Pfleif, 1989; Jiang et al., 1991).

Isolation and Analyses of Genomic and cDNA
Standard procedures, such as the isolation of genomic DNA, the construction and screening of genomic libraries in EMBL4 vectors, chromosomal walking, whole genome Southern analysis, in situ hy-
bridization to salivary gland chromosomes, and isolation of total

and poly(A) RNA were carried out essentially as described (Maniatis et al., 1982; Frei et al., 1985; Kilgher et al., 1986).

A cDNA library from poly(A) RNA from 4–8 hr old OregonR em-
byros was constructed using the Stratagene ZAP-cDNA synthesis kit (Stratagene). cDNAs were subcloned between the EcoRI (5′ end) and XhoI (3′ end) sites of the plasmid vector pBluescript SK(-). Isolation of cDNA clones was carried out according to standard procedures and following instructions from Stratagene. From a total of 7.5 × 10⁶ phage clones, 15 independent smo-cDNA clones were isolated. To clone a cDNA that includes the transcriptional start site of the smo mRNA, the 5′ RACE technique was applied to poly(A) RNA from 4–8 hr old embryos, using the Amplification RACE kit and following instructions from Clontech. A pair of independent RACE products had the same 5′ end, while a third one started two nucleo-
tides downstream of this 5′ end.

DNA sequences were determined on both strands of the smo cDNAs and the corresponding genomic DNA with a DNA sequencer model 373A using dye terminators (Applied Biosystems Inc.). DNA sequence analyses and EMBL and protein data bank searches were performed by the use of version 8.0 of the GCG sequence analysis software package (Genetics Computer Group, 1994, Wisconsin) on a Unix system.

Acknowledgments
We thank K. Martin, Y. Gonzalez, and R. Mészáros for technical assistance; J. Tennison for genetic advice; J. Tamkun for help with P-element transformation; R. Nusse for the anti-Wg antisera; T. Kornberg and R. Holmgren for the anti-En and anti-Gsb monoclonal antibodies; P. Heitzler, E. Wilder, the Tübingen and Bloomington Stock Centers for Drosophila stocks; T. Fonger for help with digital imaging; W. Boll, D. Brentrup, and T. Gutjahr for advice and help, and F. Ochsenbein for the artwork. We thank H. Noll for stimulating discussions and K. Basler and H. Noll for comments on the manu-
script. This research was supported by National Institutes of Health grant GM45396 (to J. E. H.), by Swiss National Science Foundation grants 31–26652.89 and 31–40874.94 (to M. N.), and by the Kanton Zürich.

Received June 20, 1996; revised July 9, 1996.

References


Cavener, D.R. (1987). Comparison of the consensus sequence flank-


In Drosophila, the hedgehog (hHh) gene is the first member of a large family of hedgehog (hHh) genes in vertebrates. hHh genes are expressed in a variety of tissues, including the nervous system, the heart, and the limbs. Mutations in hHh genes cause defects in organ development, such as heart defects and limb defects. The hedgehog family includes the Indian hedgehog (Ihh) gene, which is expressed in the limb and neural crest cells. Ihh signals through the Patched (Ptch) and Smoothened (Smo) proteins to activate downstream targets, such as Gli1 and Gli2, which regulate gene expression in response to hedgehog signaling.

In this study, the authors investigated the role of hedgehog signaling in the development of the Drosophila limb pattern. They used genetic and molecular techniques to identify the role of hedgehog in limb development and to understand the molecular mechanisms involved.

The authors generated a P-element vector Received June 20, 1996; revised July 9, 1996. to assay for the expression of the hedgehog gene. They used this vector to introduce a copy of the hedgehog gene into the Drosophila genome and to analyze the effect on limb development.

The authors also used immunohistochemistry and in situ hybridization to visualize the expression of hedgehog and other genes involved in limb development. They observed that hedgehog expression was upregulated in the developing limb, and that this upregulation was necessary for normal limb development.

The authors further analyzed the role of hedgehog signaling in limb development by using genetic techniques to disrupt hedgehog expression. They found that disruptions in hedgehog expression led to defects in limb development, including the loss of limb segments and the loss of normal limb morphology.

Overall, the authors provide a detailed analysis of the role of hedgehog signaling in the development of the Drosophila limb pattern. Their findings contribute to our understanding of the molecular mechanisms involved in limb development and provide a valuable model system for studying hedgehog signaling in mammals.


GenBank Accession Number

The accession number for the transcribed sequence of the smo gene reported in this article is L79947.