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An Acylatable Residue of Hedgehog Is Differentially Required in *Drosophila* and Mouse Limb Development

Jeffrey D. Lee,* Petra Kraus,† Nicholas Gaiano,* Susana Nery,* Jhumku Kohtz,* Gord Fishell,* Cynthia A. Loomis,*,† and Jessica E. Treisman*,[1]

*Skirball Institute Developmental Genetics Program, *Department of Cell Biology, and †Department of Dermatology, NYU School of Medicine, 540 First Avenue, New York, New York 10016*

The *Drosophila* Hedgehog protein and its vertebrate counterpart Sonic hedgehog are required for a wide variety of patterning events throughout development. Hedgehog proteins are secreted from cells and undergo autocatalytic cleavage and cholesterol modification to produce a mature signaling domain. This domain of Sonic hedgehog has recently been shown to acquire an N-terminal acyl group in cell culture. We have investigated the *in vivo* role that such acylation might play in appendage patterning in mouse and *Drosophila;* in both species Hedgehog proteins define a posterior domain of the limb or wing. A mutant form of Sonic hedgehog that cannot undergo acylation retains significant ability to repattern the mouse limb. However, the corresponding mutation in *Drosophila* Hedgehog renders it inactive *in vivo,* although it is normally processed. Furthermore, overexpression of the mutant form has dominant negative effects on Hedgehog signaling. These data suggest that the importance of the N-terminal cysteine of mature Hedgehog in patterning appendages differs between species.

Key Words: **Hedgehog; limb; wing; acylation; evolution.**

INTRODUCTION

Analysis of developmental events in vertebrate and invertebrate species has revealed a significant conservation of many basic developmental mechanisms, as well as a number of differences in the details. The development of appendages from imaginal discs in *Drosophila* shares many common features with vertebrate limb development (Vogt and Duboule, 1999). The posterior compartment of the *Drosophila* wing disc, defined by the presence of the transcription factor Engrailed (En), expresses Hedgehog (Hh), a secreted signaling protein. Hh acts directly to specify the central region of the wing (Mullor *et al.,* 1997; Strigini and Cohen, 1997) and also stimulates cells just anterior to the compartment boundary to express the TGF-β family member Decapentaplegic (Dpp; Basler and Struhl, 1994; Tabata and Kornberg, 1994). Dpp then acts as a long-range morphogen to pattern the entire wing (Lecuit *et al.,* 1996; Nellen *et al.,* 1996). The posterior region of the vertebrate limb bud, known as the zone of polarizing activity (ZPA), likewise secretes the Hh homologue Sonic hedgehog (Shh), which acts to pattern the limb along the anteroposterior axis (Kraus *et al.,* 2001; Riddle *et al.,* 1993; Wang *et al.,* 2000; Yang *et al.,* 1997). There is no evidence for a compartment boundary anterior to the domain of Shh expression; however, Shh activates the expression of the Dpp homologues bone morphogenetic protein 2 (BMP2) and BMP7, which further contribute to limb patterning (Drossopoulou *et al.,* 2000; Duprez *et al.,* 1996; Francis *et al.,* 1994; Laufer *et al.,* 1994; Yang *et al.,* 1997; Zhang *et al.,* 2000; Zuniga *et al.,* 1999).

In addition to their functions in limb development, proteins of the Hh family function as positional cues in a wide variety of other developmental contexts. In *Drosophila* these include embryonic segmentation and the progressive differentiation of photoreceptors in the eye disc (Ingham, 1994). In vertebrates Shh is required for the patterned

[1] To whom correspondence should be addressed. Fax: (212) 263-7760. E-mail: treisman@saturn.med.nyu.edu.

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specification of ventral cell fates in the neural tube and in the adjacent somites (Chiang *et al.*, 1996; Fan *et al.*, 1995; Roelink *et al.*, 1995). All Hh family members are thought to act by binding to homologues of the transmembrane protein Patched (Ptc). In the absence of Hh binding, Ptc inactivates the transmembrane protein Smoothened (Smo; Alcedo and Noll, 1997; Chen and Struhl, 1998; Marigo *et al.*, 1996; Murone *et al.*, 1999; Stone *et al.*, 1996); this inhibition of Smo is relieved by Hh binding to Ptc, allowing Smo to transduce the Hh signal. This results in the production of an active form of the transcription factor Cubitus interruptus (Ci) or its homologues encoded by the vertebrate Gli genes (Aza-Blanc *et al.*, 1997; Chen *et al.*, 1999a; Methot and Basler, 1999; Ohlmeyer and Kalderon, 1998; Sasaki *et al.*, 1999; Wang *et al.*, 2000).

Hh is initially translated as a 45-kDa preprotein with an internal signal sequence near the N-terminus. In addition to removal of this signal sequence, the full-length protein is cleaved internally by the autoproteolytic activity of the C-terminal domain, resulting in a mature N-terminal 19-kDa ligand (Lee *et al.*, 1994). As a result of the autocatalytic mechanism, the mature protein acquires a cholesterol moiety linked to the C-terminal amino acid (Porter *et al.*, 1995, 1996a,b). The function of this modification may be to tether Hh to the cell surface and thereby limit its diffusibility; a form of Hh that lacks cholesterol shows increased diffusibility and activity (Porter *et al.*, 1996a). The Dispatched protein, which has a sterol-sensing domain, is specifically required to release cholesterol-modified Hh from cells (Burke *et al.*, 1999). In addition, Hh requires heparan sulfate proteoglycan synthesis by the product of the *tout velu* gene in order to move from cell to cell (Bellaiche *et al.*, 1998; The *et al.*, 1999). Although Shh has an N-terminal, rather than internal, signal sequence, it undergoes the same autoproteolytic cleavage as *Drosophila* Hh to produce a mature N-terminal protein carrying a cholesterol moiety (Porter *et al.*, 1995, 1996b).

In addition to the cholesterol modification, human and rat Shh proteins produced in tissue culture have a fatty acid attached to cysteine-24, which is the N-terminal amino acid of the mature signaling protein (Pepinsky *et al.*, 1998). Full-length human Shh has been shown to be modified by the addition of palmitic acid, whereas the N-terminal domain of rat Shh can be modified by any of several different fatty acids (Pepinsky *et al.*, 1998). Although the thiol group of Cys-24 is essential for palmitoylation of human Shh, palmitate is attached to the N-terminal α-amino group rather than forming a thioester linkage (Pepinsky *et al.*, 1998). Acylation appears to be required to potentiate human Shh activity on C3H10T1/2 cells (Pepinsky *et al.*, 1998; Williams *et al.*, 1999) as well as in some contexts *in vivo* (Kohtz *et al.*, 2001). Unacylated Shh produced in *Escherichia coli* can induce the formation of ventral neuronal cell types in chick spinal cord and forebrain explants, but is much less active on mouse forebrain explants than acylated Shh produced in insect cells (Kohtz *et al.*, 2001). Thus unacylated Shh appears to function as a hypomorphic form of the protein in some contexts.

To test whether acylation is important for Hh signaling in *Drosophila* or mouse appendage patterning, we have constructed forms of *Drosophila* Hh (C84S-Hh) and human Shh (C24S-Shh) that cannot be acylated because the cysteine to which the acyl group is normally attached has been mutated to a serine. In the mouse limb, C24S-Shh appears to have reduced patterning activity compared to wild-type Shh. However, C84S-Hh has no detectable Hh activity in *Drosophila* and can interfere with the function of endogenous Hh when expressed at high levels. This mutation does not appear to affect processing or secretion of the protein, and C24S-Shh and unacylated wild-type Shh have equivalent activities on forebrain explants *in vitro* (Kohtz *et al.*, 2001). Thus our results suggest that this cysteine residue is required throughout evolution, perhaps as a substrate for acylation, although its impact on Hh signaling varies across species.

MATERIALS AND METHODS

Retroviral Preparation and Injection

Shh and C24S-Shh sequences were inserted into a modified MLV retroviral vector (CLE) immediately upstream of an IRES linked to the placental alkaline phosphatase (PLAP) reporter gene to permit detection of infected cells (Gaiano *et al.*, 1999). The replication-defective retroviral vectors were pseudotyped with the VSV-G protein and injected into the amniotic sac at titers of $2–4 \times 10^7$ cfu/ml using ultrasound-guided imaging, as described (Gaiano *et al.*, 1999). Injections were performed at 8.5 to 9.5 dpc (days postcoitum), just prior to initiation of fore- and hindlimb outgrowth, respectively (Gaiano *et al.*, 1999; Turnbull *et al.*, 1995).

Mouse Tissue Preparation and Analysis

Mouse embryos were delivered by caesarian dissection at 12.5 or 18.5 dpc and fixed in 4% paraformaldehyde overnight. After fixation, the embryos were washed in phosphate-buffered saline with 0.1% Tween 20. Endogenous alkaline phosphatase was inactivated by heating for 20 min at 70°C. The embryos were then equilibrated in NTMT buffer (0.1 M Tris, pH 9.5/0.05 M $MgCl_2$/0.1 M NaCl/0.05% levamisole/0.1% Tween 20) and subsequently stained for alkaline phosphatase activity, representing viral gene expression (Gaiano *et al.*, 1999). Scattered clusters of reporter gene expressing cells were detected in the presumptive skin. Subsequent RNA *in situ* hybridization analysis using a *Hoxd13* probe (Dolle *et al.*, 1989) was performed on some of the embryonic limbs stained, essentially as described (Loomis *et al.*, 1998). In these double-labeling experiments, two different alkaline phosphatase substrates were used to produce distinctly colored reaction products. Alcian blue and alizarin red stained skeletal preparations of 18.5 dpc embryos were performed as described (Lufkin *et al.*, 1992).

Drosophila Genetics

Alleles and transgenic lines used were hh^{ts2} (Ma *et al.*, 1993), hh^{AC} (Lee *et al.*, 1992), ptc^{s2} (Flybase), *dpp-lacZ* (Blackman *et al.*,

1991), *da*-GAL4, *ptc*-GAL4 (Wodarz *et al.*, 1995), *omb*-GAL4 (Lecuit *et al.*, 1996), *en*-GAL4 (Flybase), and *ey*-GAL4 (Hazelett *et al.*, 1998). pUAS-*hh* was generated by cloning a *Kpn*I/*Xba*I fragment containing the entire coding region of *hh* into the pUAST vector. The C84S mutation was made by site-directed mutagenesis using an antisense oligonucleotide with the sequence 5′-CCAGGACCGGAGCTGTGAGCC-3′ and the Sculptor kit (Amersham). The wild-type TGC codon, which specifies cysteine, was replaced by a TCC codon, which specifies serine. C84S-*hh* was then inserted into pUAST using *Kpn*I and *Xba*I. Both constructs were confirmed by sequencing. Constructs were injected into *white*$^-$ embryos using standard methods and several independent transformants for each construct were generated. All insertions used in this study are on the third chromosome and were balanced over TM6B.

Immunohistochemistry and Microscopy

Imaginal discs were dissected in PBS and fixed on ice for 30 min in 4% formaldehdye/1× PEM (0.1 M Pipes, pH 7.0/2 mM $MgSO_4$/1 mM EGTA). Primary antibody staining was done overnight at 4°C in PBS/0.2% Triton X-100/10% normal donkey serum. Antibodies were used at the following dilutions: rabbit α-Nhh-1 (gift of T. Tabata) was used at 1:2000, mouse α-Ptc (gift of I. Guerrero) was used at 1:200, rat α-Ci monoclonal 2A1 (Motzny and Holmgren, 1995; gift of R. Holmgren) was used at 1:1, and mouse α-En monoclonal 4D9 (gift of S. DiNardo) was used at 1:1. Donkey secondary antibodies conjugated to FITC or Texas red (Jackson Laboratories) were diluted 1:200 in PBS/0.2% Triton X-100/10% normal donkey serum and incubated 2 h at 4°C. Antibody labeling was detected on a Leica DMRBE confocal microscope. LacZ activity was detected by X-gal staining. Adult wings were mounted in Canada balsam:methyl salicylate (2:1) and visualized using a Zeiss Axioplan microscope.

Western Blot Analysis

Flies carrying a *da*-GAL4 transgene were crossed to flies carrying either UAS-*hh* or UAS-*C84S-hh* transgenes; *da*-GAL4 is expressed ubiquitously in the embryo. Embryos were harvested at 2–8 h after egg laying in PBS/0.2% Triton X-100 + a protease inhibitor cocktail (Boehringer-Mannheim No. 1836170). Embryos were pelleted and resuspended in an equal volume of 2× Laemmli buffer + protease inhibitors. The embryos were homogenized by repeated passes with a micropestle, followed by a 5-min incubation at 100°C, followed by more passes with the pestle. Samples were stored at −20°C until use. Approximately 20 embryo equivalents per lane were electrophoresed on a 12% acrylamide denaturing gel and transferred overnight to nitrocellulose. Primary and secondary antibody incubations were as previously described (Therond *et al.*, 1996); rabbit α-Nhh-1 was diluted 1:5000.

Cuticle Preparations

Flies of the genotype hh^{ts2}; *en*-GAL4/SM6.TM6B were crossed to flies of the genotype hh^{AC}, UAS-(*hh* or *C84S-hh*)/TM6B or hh^{AC}/TM6B. The crosses were kept at 29°C to inactivate the hh^{ts2} allele. Unhatched embryos were collected at 12–24 h after egg laying and dechorionated in bleach. The cuticles were fixed for 60 min at 60° in acetic acid:glycerol at 4:1 and mounted overnight at 60°C in Hoyers:lactic acid:H_2O, 2:1:1.

RESULTS

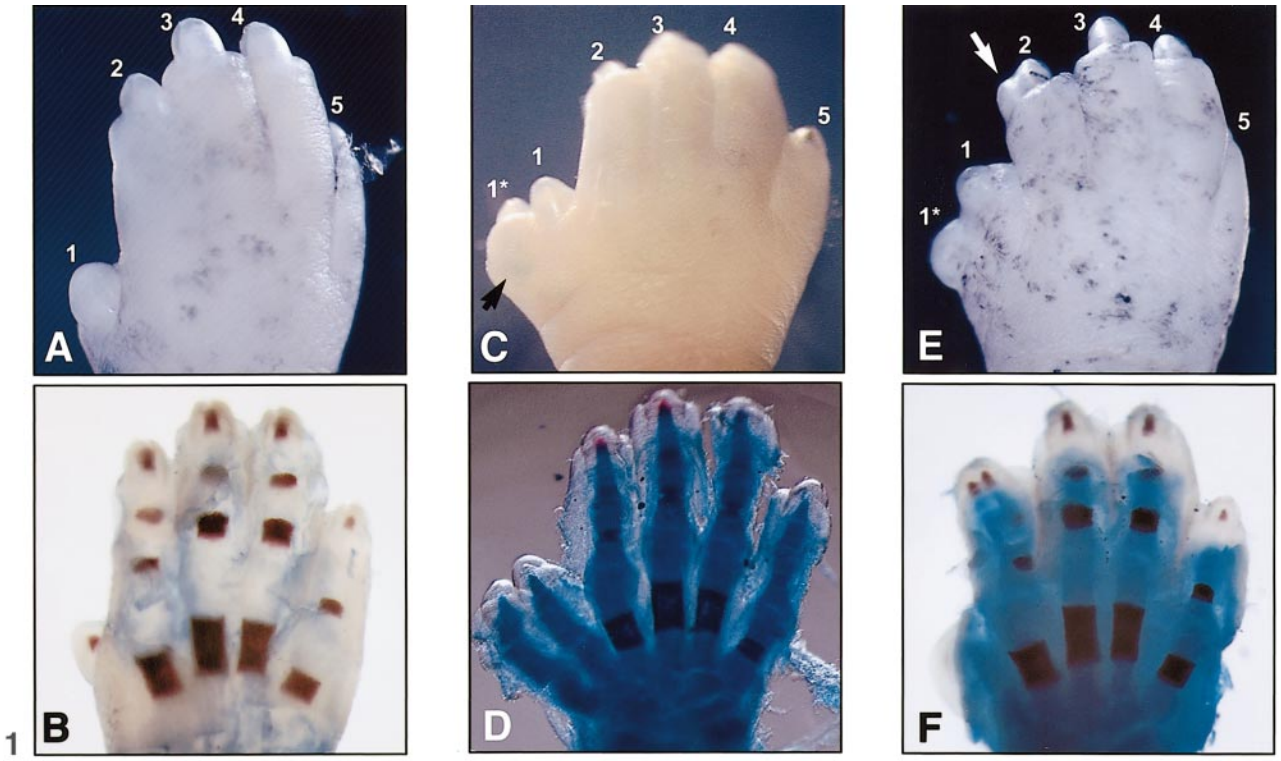
Acylation Is Not Essential for Shh to Induce Polydactyly in Mouse Limbs

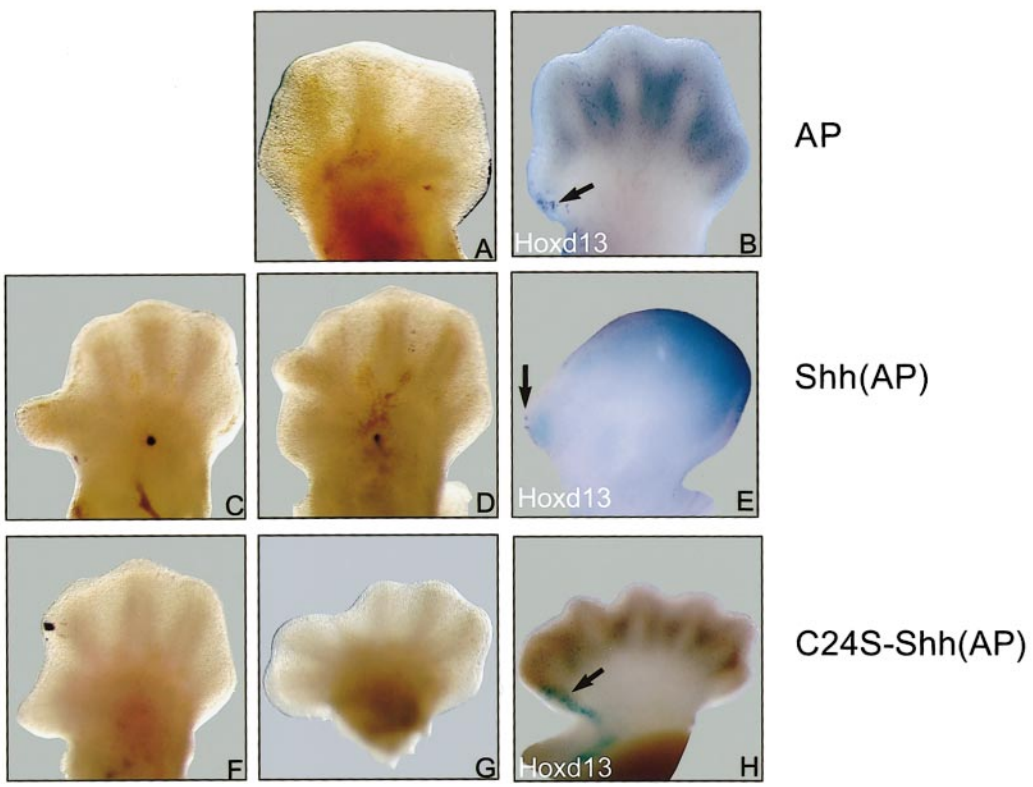
A mutant form of human Shh, which cannot be acylated because cysteine-24 is changed to serine (C24S-Shh-N), is at least 800-fold less active in inducing alkaline phosphatase activity in C3H10T1/2 cells than wild-type acylated Shh-N and can antagonize the effect of Shh-N on these cells (Williams *et al.*, 1999). In addition, C24S-Shh has much less ability to ventralize the embryonic mouse forebrain than acylated Shh does (Kohtz *et al.*, 2001). To further evaluate the impact of acylation on other aspects of Shh function during mouse development, we used retroviral vectors to misexpress full-length wild-type and C24S-Shh in developing limb buds. Shh and C24S-Shh sequences were inserted immediately upstream of an internal ribosome entry site (IRES) followed by a PLAP gene to allow easy detection of infected cells.

Retroviral-mediated misexpression of wild-type *Shh* in the early mouse limb bud resulted in polydactyly (Figs. 1C, 1D, and 2D) and/or proximal deviation of the anterior digits (Fig. 2C) resembling the hand signal of a hitchhiker. A strong correlation of such phenotypes with retroviral infection in the distal and anterior regions of the limb bud was observed (Figs. 2C–2E). These results were consistent with previous studies demonstrating digit duplication and al-

FIG. 1. Limbs infected with Shh or C24S-Shh-expressing virus display anterior polydactyly. (A, C, E) show whole-mount paws stained for alkaline phosphatase activity and (B, D, F) subsequent alcian blue and alizarin red stained skeletal preparations from 18.5 dpc embryos injected with (A, B) control and (C, D) Shh expressing- and (E, F) C24S-Shh expressing retroviruses between 8.5 and 9.5 dpc. A completely formed ectopic digit 1 is present in both the Shh and C24S-Shh-expressing limbs (1*) as well as a bifurcated digit 2 in the C24S-Shh-expressing limb (arrow). Although the degree of anterior duplication was variable, such duplications were never observed in limbs injected with control virus. At 18.5 dpc, retroviral-mediated gene expression was still detectable in the skin, confirming adequate initial infection. Retroviral alkaline phosphatase activity was most frequently noted in the epidermis (ectoderm-derived), but occasional expression in the dermis (mesoderm-derived) was also observed (C, arrow).

FIG. 2. Anterior Shh or C24S-Shh induces molecular and morphological alterations at early stages of limb development. All panels show whole mounts of 12.5 dpc limbs infected with (A, B) control PLAP-expressing, (C–E) Shh-expressing, or (F–H) Shh-C24S-expressing retroviruses. (C–H) Phenotypes ranging from (C, F) digit deviation (hitchhiker-like handplate) to (D, G) formation of extra anterior digits.





(A, B) Control limbs expressing PLAP alone show no anterior plate abnormalities. (E, H) Anterior induction of the Shh downstream target *Hoxd13* in response to ectopic Shh/Shh-C24S expression. Viral expression was demonstrated by staining for PLAP activity (arrows, purple in B and E and turquoise in H) prior to performing the RNA *in situ* hybridization for the *Hoxd13* mRNA (blue in B and E and brown in H). (B) Note that massive infection with control virus does not cause upregulation of *Hoxd13* in the most anterior region of the hand plate.

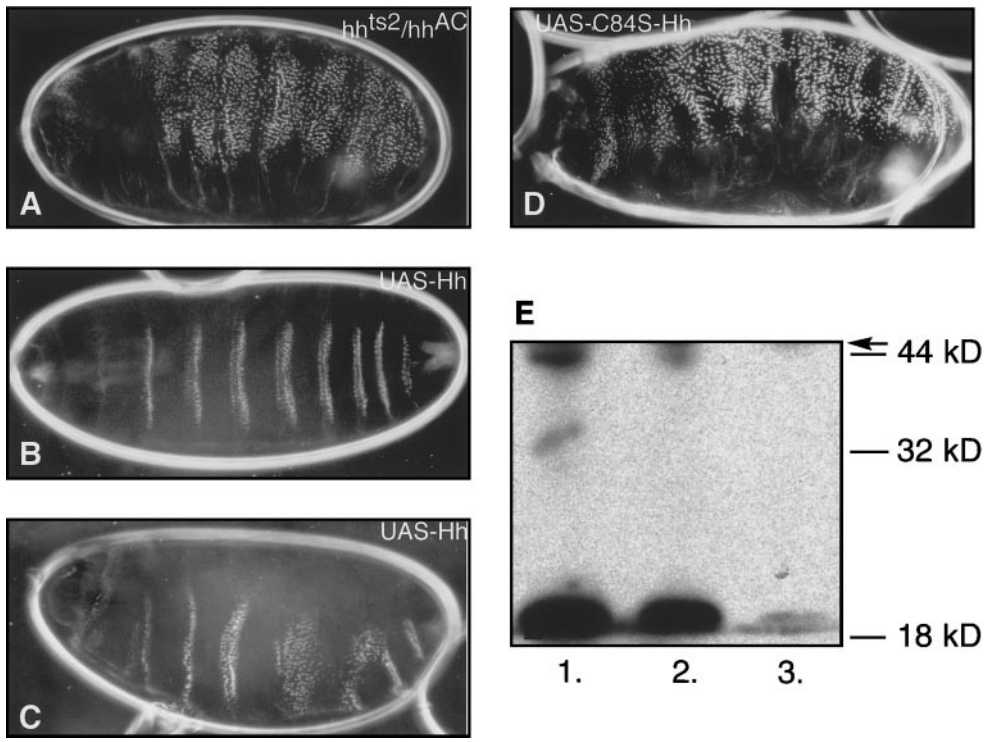


FIG. 3. Activity and processing of C84S-Hh. (A–D) show cuticles of embryos grown at 29°C. (A) hh^{AC}/hh^{ts2}. (B, C) hh^{AC}/hh^{ts2}; UAS-*hh*/*en*-GAL4. UAS-*hh* rescues the lack of endogenous *hh* activity completely (B) or partially (C). (D) hh^{AC}/hh^{ts2}; UAS-*C84S-hh*/*en*-GAL4. C84S-Hh fails to rescue *hh* mutants, resulting in a severe segment polarity phenotype. (E) Western blot of protein extracts from embryos expressing full-length wild-type Hh (1), C84S-Hh (2), or *da*-GAL4 alone (3) probed with α-Hh. Both the wild-type and the C84S-Hh constructs express protein that is processed to the correct molecular weight for mature Hh-N.

tered anterior–posterior patterning when ectopic Shh is delivered to the anterior limb mesenchyme (Chen *et al.*, 1999b; Liu *et al.*, 1998; Riddle *et al.*, 1993; Yang *et al.*, 1997) and loss of anterior–posterior expansion of the handplate in the absence of Shh (Kraus *et al.*, 2001). In contrast, Shh overexpression by cells in the posterior limb where Shh is expressed endogenously or ectopic expression by cells in more proximal limb domains had no apparent impact on early limb patterning (Fig. 2C and data not shown). Thus, as expected, the ability of Shh to influence limb development was dependent on its location along the anterior–posterior axis. The phenotypes, however, did not appear to require mesenchymal expression of Shh. Indeed, since retroviral injection after 9.0 dpc infects almost exclusively the surface ectoderm, the ectopic anterior outgrowths were most frequently induced by Shh-expressing cells located in close proximity to or within the apical ectodermal ridge (AER), an ectoderm-derived structure (Fig. 1).

Mutant C24S-Shh produced similar results in the limb bud. Ectopic anterior expression of C24S-Shh in the ectoderm induced polydactyly (Figs. 1E, 1F, and 2G) and deviation of the anterior digits (Fig. 2F). Also, similar to wild-type Shh, ectopic proximal or posterior C24S-Shh had no overt effects on limb patterning (data not shown). Morphological analysis of 327 infected limbs indicated that both acylated and unacylated forms of Shh induced a similar spectrum of phenotypes (Table 1), although C24S-Shh appeared less active than wild type. In contrast to limbs infected with Shh and C24S-Shh-containing vectors, limbs infected with control retroviral vectors expressing only the PLAP reporter gene did not develop the anterior handplate phenotypes characteristic of Shh- and C24S-Shh-expressing limbs, even when robust AP reporter expression was detected anteriorly (Figs. 2A and 2B).

We also examined the effect of wild-type Shh and C24S-Shh on a Shh target gene. We chose to look at *Hoxd13*, which requires Shh signaling for expansion and robust expression within the forming handplate (Kraus *et al.*, 2001, and references therein). We have further demonstrated that *Hoxd13* is strongly upregulated in *Shh* mutant limb buds following infection with Shh-expressing virus and is therefore a useful readout of Shh activity (P. Kraus, unpublished data). *Hoxd13* is normally not expressed in the anterior-most mesenchyme of 12.5 dpc limb buds. However, in response to ectopic anterior expression of either Shh or C24S-Shh but not of the PLAP reporter, *Hoxd13* expression was induced in the most anterior domain of the developing handplate (Figs. 2E and 2H).

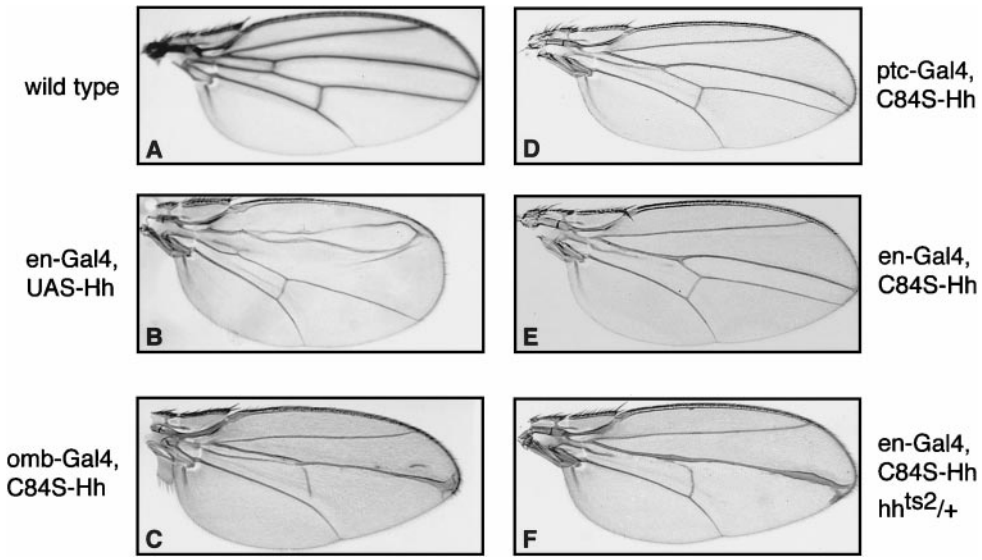


FIG. 4. C84S-Hh dominantly represses the formation of Hh-dependent structures in the wing. All panels show adult wings. (A) Wild type. (B) *en*-GAL4/UAS-*hh*. The L3–L4 intervein region is expanded. (C) *omb*-GAL4/+; UAS-*C84S-hh*/+. The L3–L4 intervein region is reduced or absent. (D) *ptc*-GAL4/UAS-*C84S-hh*. L3 and L4 are fused proximally; the anterior crossvein is lost. (E) *en*-GAL4/UAS-*C84S-hh*. The L3–L4 intervein region is reduced or lost. (F) hh^{ts2}/+; *en*-GAL4/UAS-*C84S-hh*, kept at 29°C. Reduction of endogenous Hh production enhances the intervein loss phenotype (compare to E).

Mutation of the N-Terminal Cysteine of the Signaling Domain of Drosophila Hedgehog Abolishes Its Activity

In order to test whether acylation of the corresponding cysteine of *Drosophila* Hh could affect the activity of the protein, we constructed a mutant *hh* cDNA encoding serine instead of cysteine at position 84 (referred to hereafter as C84S-Hh). Cys-84 is the N-terminal residue of the mature Hh protein, corresponding to Cys-24 in Shh, and is followed by a conserved sequence of amino acids (Fietz *et al.*, 1994). We used the GAL4-UAS system (Brand and Perrimon, 1993) to express wild-type and C84S-Hh in transgenic flies. To test whether C84S-Hh could replace wild-type Hh, we expressed C84S-Hh in the normal Hh pattern, under the control of *engrailed (en)*-GAL4, in flies transheterozygous for null and temperature-sensitive alleles of *hh*. At the restrictive temperature, embryos of the genotype hh^{ts}/hh^{AC} display a severe segment polarity phenotype in which the naked cuticle is lost (Fig. 3A). When a wild-type Hh transgene was expressed using *en*-GAL4 in hh^{ts}/hh^{AC} embryos,

TABLE 1
Summary of Shh/C24S-Shh-Induced Limb Phenotypes

Transgene		Phenotype				
		Extra digits	Hitchhiker (HH)	Extra + HH	Truncated	Total
PLAP	total	—	—	—	2 (6%)	35
C24S-Shh	total	23 (19%)	6 (5%)	3 (2%)	5 (4%)	124
Shh	total	62 (31%)	17 (8%)	12 (6%)	11 (5%)	203

Note. The percentages of each class of phenotype observed are described for each experiment. Anterior polydactyly and anterior handplate deviations (hitchhiker-like digits) were observed only in experimental (Shh and C24S-Shh) limbs. In general, C24S-Shh induced a lower percentage of extra digits than did wild-type Shh, consistent with C24S-Shh functioning as a hypomorphic signaling molecule. Rare distal truncations, ranging from mild to almost complete loss of structures, were observed in approximately 5% of both control and experimental limbs. Such phenotypes resembled the limb reduction defects noted in a small number of human fetuses after chorionic villous sampling (Firth, 1997) and were likely due to global physiological alterations, such as a transient bradycardia and hypoxia, caused by the injection procedure itself (MacIntyre *et al.*, 1995).

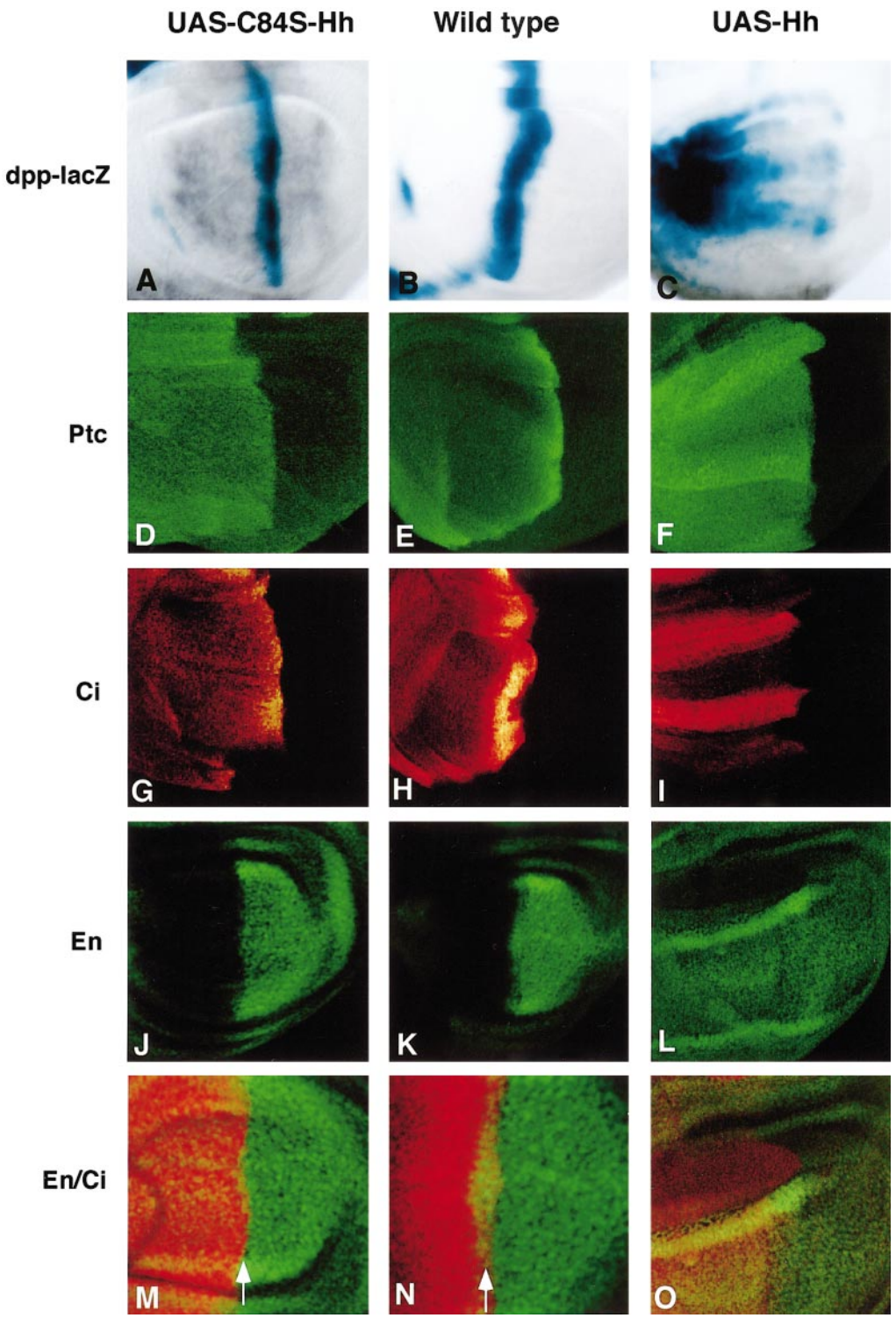


FIG. 5. C84S-Hh represses the expression of Hh target genes in the wing imaginal disc. All panels show third instar wing disc pouches with anterior to the left and dorsal up. (A, D, G, J, M) *omb*-GAL4/+; UAS-*C84S-hh*/+. (B, E, H, K, N) Wild-type expression patterns. (C, F, I, L, O) *omb*-GAL4/+; UAS-*hh*/+. (A–C) *dpp-lacZ* expression revealed by X-gal staining is shown in blue. Expression of the transgene (A, C) or endogenous Hh (B) is revealed by Hh antibody staining (brown). (D–F) Ptc antibody staining. (G–I) Ci antibody staining. (J–L) En antibody staining. (M–O) High magnification of the wing pouch stained for Ci (red) and En (green). En expression anterior to the AP compartment boundary overlaps Ci (N, arrow); this overlap is lost when C84S-Hh is expressed (M, arrow). Late overexpression of Hh in the anterior compartment induces *en* expression; En protein transcriptionally represses *ci* (I, L, O).

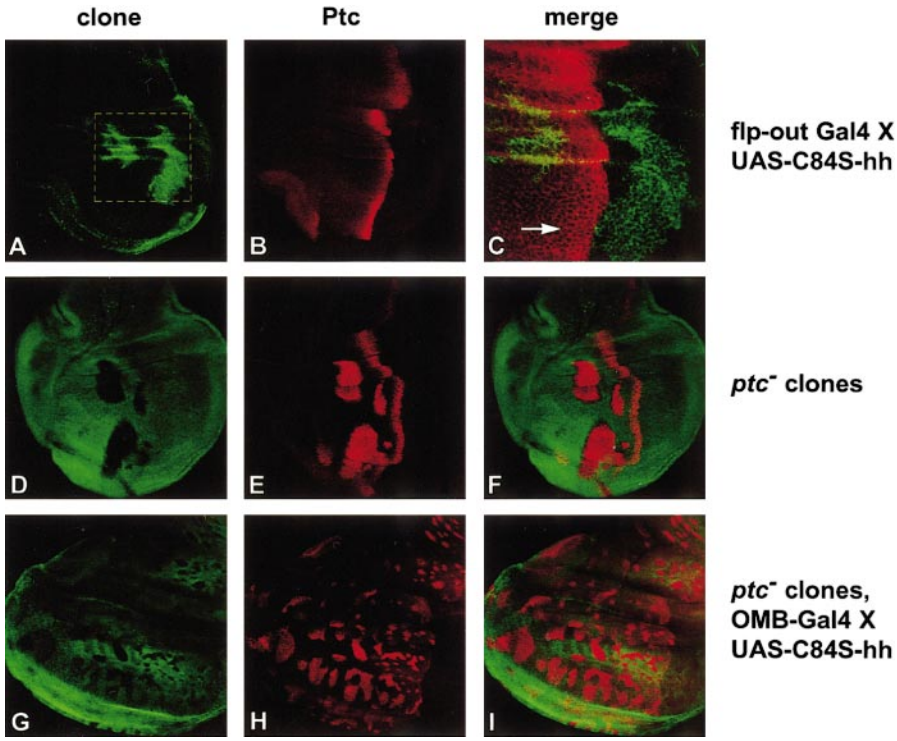


FIG. 6. The dominant negative activity of C84S-Hh is nonautonomous and requires Ptc. All panels show third instar wing disc pouches with anterior to the left and dorsal up. (A–C) *Act*<*CD2*<GAL4, *hs*-FLP122/+; UAS-*C84S-hh*/UAS-*lacZ*. (D–F) *hs*-FLP122/+; FRT42.*ptc*^{s2}/FRT42.*arm*-lacZ. (G–I) *omb*-GAL4, *hs*-FLP122/+;FRT42.*ptc*^{s2}/FRT42.*arm*-lacZ; UAS-*C84S-hh*/+. (A–C) Clones expressing C84S-Hh are positively labeled by antibodies to β-galactosidase (green in A,C); Ptc protein (red in B, C) is reduced near the clones (arrow in C). (C) An enlargement of the region boxed in (A). (D–I) *ptc* clones are revealed by loss of β-galactosidase staining (green in D, F, G, I); Ptc protein (red in E,F, H, I) is upregulated in the clones, even when C84S-Hh expression is driven by *omb*-Gal4 and downregulates Ptc outside the clones (G–I).

most of the cuticles examined were wild type or showed a mild segment polarity phenotype (Figs. 3B and 3C, Table 2). Thus this *en*-GAL4 line can direct the production of sufficient protein to rescue Hh signaling. In contrast, when C84S-Hh was expressed in *hh*^{ts}/*hh*^{AC} embryos, the mutant embryos failed to hatch and showed a segment polarity phenotype that was indistinguishable from the *hh*^{ts}/*hh*^{AC} phenotype in the absence of any rescuing construct (Fig. 3D, Table 2). The lack of rescue by C84S-Hh was observed with several independent UAS lines, all of which could strongly

TABLE 2

Summary of Cuticle Rescue Experiment

Genotype	Wild-type cuticle	*hh* null cuticle	Intermediate	Total
en-Gal4; *hh*^{ts2}/SM6.TM6B				
X UAS-hh^{4j2}, *hh*^{AC}/TM6B	238	14	21	273
Percentage of total	87.2%	5.1%	7.7%	
en-Gal4; *hh*^{ts2}/SM6.TM6B				
X UAS-C84S-Hh^{D7}, *hh*^{AC}/TM6B	151	72	0	223
Percentage of total	67.7%	32.3%	0	

Note. Cuticles of all embryos in each cross were collected prior to hatching, at 16 h after egg laying. The expected proportion of *hh* null cuticles would be 25% in both experiments. The higher number observed following C84S-Hh expression could be due to dominant negative activity in some embryos. The "intermediate" class of cuticle phenotype is shown in Fig. 3C.

express ectopic Hh detected by antibody staining (data not shown).

The inability of C84S-Hh to rescue the *hh* mutant phenotype could be caused by an effect of the C84S mutation on protein stability or processing. To test this, we performed a Western blot analysis of protein extracts from embryos overexpressing the C84S-Hh or wild-type Hh constructs under the control of the ubiquitous *daughterless* (*da*)-GAL4 driver. Using a polyclonal antiserum against Hh protein, we found that the abundance of the 19-kDa N-terminal fragment of Hh was greatly increased in protein extracts from embryos expressing either wild-type or C84S Hh (Fig. 3E). The ratio of unprocessed to processed Hh appeared the same in embryos overexpressing either wild-type Hh or C84S-Hh. Thus the C84S mutation abolishes the function of Hh without affecting its processing.

C84S-Hh Has a Dominant-Negative Effect on Hh Signaling

To test whether any residual function of C84S-Hh could be detected in a gain of function assay, we used a series of GAL4 lines to express either C84S-Hh or wild-type Hh in a variety of embryonic and larval tissues (see Materials and Methods). While misexpression of wild-type Hh caused clear patterning defects often leading to lethality, C84S-Hh displayed no Hh gain of function activity in any of the tissues we tested. We found that C84S-Hh misexpression caused a visible phenotype only in the developing wing and the ocelli (Fig. 4 and data not shown).

In the wing imaginal disc, Hh is produced by cells of the posterior compartment and signals to cells at the anterior–posterior (AP) border (Basler and Struhl, 1994; Tabata and Kornberg, 1994). Hh directly patterns the wing in the vicinity of the AP border, corresponding to the L3–L4 intervein region in the adult (Mullor *et al.*, 1997; Strigini and Cohen, 1997); Hh also indirectly patterns the remainder of the wing through the induction of *dpp* expression at the AP border (Basler and Struhl, 1994; Zecca *et al.*, 1995). Overexpression of wild-type Hh in the posterior compartment of the wing disc using *en*-GAL4 resulted in an expansion of the anterior compartment (Fig. 4B). However, when we overexpressed C84S-Hh in the posterior compartment we observed a loss of tissue in the region surrounding the compartment boundary, between veins L3 and L4, and the partial fusion of these veins (Fig. 4E). This phenotype resembles loss of function mutations in the genes *fused* and *cubitus interruptus* (*ci*), which are required for Hh signal transduction in the wing (Busson *et al.*, 1988; Methot and Basler, 1999). We also misexpressed Hh and C84S-Hh with *optomotor blind (omb)*-GAL4, which is expressed in a broad domain of the wing disc centered on the AP compartment border (Grimm and Pflugfelder, 1996). Expression of wild-type Hh using *omb*-GAL4 caused an expansion of the L3–L4 region (data not shown); in contrast, C84S-Hh expression using *omb*-GAL4 resulted in an even stronger loss of L3–L4 tissue and partial or complete fusion of veins 3 and 4 (Fig. 4C). These results suggest that, rather than mimicking wild-type Hh, C84S-Hh may interfere with the endogenous Hh signal. Likewise, when C84S-Hh was misexpressed in the ocellar region of the eye-antennal imaginal disc using *eyeless*-GAL4, formation of the ocelli was blocked (data not shown); ocellar development requires Hh signaling (Royet and Finkelstein, 1996).

C84S-Hh may directly compete with wild-type Hh for binding to the Ptc receptor (Chen and Struhl, 1998; Marigo *et al.*, 1996); alternatively, it could simply interfere with the production of wild-type Hh. To address this question we expressed C84S-Hh in anterior wing disc cells that do not normally produce Hh protein, using *ptc*-GAL4. *ptc* is upregulated in anterior cells responding to Hh; however, *ptc* is not expressed in the posterior cells that produce Hh (Alexandre *et al.*, 1996; Schwartz *et al.*, 1995). C84S-Hh expression under the control of *ptc*-GAL4 also reduced the amount of L3–L4 tissue produced (Fig. 4D). This result suggests that C84S-Hh interferes with Hh signaling in the extracellular environment and not at the level of intracellular processing.

If C84S-Hh acts by competing with wild-type Hh, the severity of the phenotype should depend on the relative amounts of C84S-Hh and wild-type Hh present. We tested this by altering the ratio of wild type to mutant protein being expressed. When C84S-Hh was expressed in flies heterozygous for *hh*, the reduction of the L3–L4 region was enhanced (Figs. 4E and 4F), thus decreasing the dosage of wild-type Hh relative to C84S-Hh exacerbates the dominant negative phenotype.

C84S-Hh Reduces the Expression of Hh Target Genes in the Wing Disc

To further investigate the disruption of Hh signaling by C84S-Hh, we examined the expression of Hh target genes in third instar wing imaginal discs in which C84S-Hh was expressed. *dpp-lacZ* and *ptc* are normally upregulated at the AP border in response to Hh (Figs. 5B and 5E). When wild-type Hh was expressed using *omb*-GAL4, *dpp-lacZ* expression and high levels of Ptc protein were detected throughout most of the anterior compartment (Figs. 5C and 5F). When C84S-Hh was expressed, *dpp-lacZ* expression along the AP border was reduced (Fig. 5A). However, this reduction was rather mild, which may explain why the overall size of the wing and its patterning outside of the L3–L4 region was unaffected. Ptc upregulation was also greatly reduced when C84S-Hh was expressed (Fig. 5D).

The zinc-finger protein Ci is expressed throughout the anterior compartment and is constitutively cleaved to produce a truncated protein that represses Hh target genes (Aza-Blanc *et al.*, 1997); this cleavage event is suppressed in cells receiving the Hh signal at the AP border, resulting in a stripe of the full-length form of Ci that can be detected with an antibody to a C-terminal epitope (Motzny and Holmgren, 1995; Fig. 5H). Stabilization of the full-length Ci protein was decreased when C84S-Hh was expressed (Fig.

5G). When wild-type Hh was expressed, full-length Ci appeared to be expanded but at a relatively low level (Fig. 5I). This may be explained by the effect of Hh on *en*, a negative regulator of Ci (Schwartz *et al.*, 1995). *en* expression is expanded across the AP border in late third instar in response to Hh (Hidalgo, 1994; Strigini and Cohen, 1997; Figs. 5K and 5N). En represses *ci* transcription, resulting in a narrow stripe of reduced Ci staining at the AP border (Fig. 5N). The anterior expansion of En was prevented by C84S-Hh expression (Figs. 5J and 5M); in contrast, high levels of En were detected in the anterior compartment when wild-type Hh was misexpressed (Figs. 5L and 5O).

Our results in adult wings suggested that C84S-Hh expressed in either the Hh-producing or -receiving cells could interfere with Hh function (Figs. 4D and 4E). To confirm that C84S-Hh could act nonautonomously on Hh target genes, we expressed C84S-Hh in labeled clones of cells near the AP border of the wing imaginal disc and examined Hh target gene expression. Ptc expression was reduced in cells adjacent to and up to a few cell diameters from C84S-Hh-expressing clones (Figs. 6A–6C). Clones contained entirely within the posterior compartment were able to reduce Ptc expression in nearby anterior cells (Fig. 6C), confirming that the C84S-Hh protein can be secreted and suggesting that it competes with wild type Hh in the extracellular space.

A likely target for the dominant negative activity of C84S-Hh is the Hh receptor Ptc. *ptc* mutant cells upregulate Hh target genes; if C84S-Hh competes with wild-type Hh for binding to Ptc, then it should have no dominant negative effect on cells lacking *ptc* activity. However, if C84S-Hh acts independently of Ptc, for example, by directly inhibiting Smo activation or by inhibiting another, uncharacterized, Hh receptor, it should still block Hh signaling in *ptc* mutant cells. We investigated this by producing clones of cells mutant for *ptc* in wing imaginal discs that overexpressed C84S-Hh in the *omb* pattern. Ptc protein was upregulated in *ptc* mutant clones both in wild-type wing discs (Figs. 6D–6F) and in wing discs overexpressing C84S-Hh (Figs. 6G–6I). Combined with the fact that the dominant negative activity of C84S-Hh is nonautonomous, this result suggests that C84S interferes with the binding of wild-type Hh to Ptc or to a protein upstream of Ptc.

In summary, these results indicate that, while C84S-Hh is processed to the mature signaling protein, it has no positive signaling activity. However, it is able to interfere at the extracellular level with endogenous Hh signaling in the wing disc. This suggests that C84S-Hh may still be able to interact with the Hh signaling pathway; this interaction is most likely to be at the level of the Hh receptor Patched. The effects of C84S-Hh are seen in the region of the wing that requires the highest levels of Hh to develop normally, and the *en* gene, which requires high levels of Hh for its expression, is most strongly affected. These data extend the observation that C24S-Shh-N is a dominant negative protein in tissue culture (Williams *et al.*, 1999) to show that the same mutation in full-length *Drosophila* Hh gives it dominant negative activity *in vivo*.

DISCUSSION

Previous studies have shown that interpretation of the Hh signal depends on the amount of Hh that is sensed by the receiving cell, as well as by the developmental history of the cell, which may influence the levels of components of the Hh signaling pathway. The timing and duration of Hh production can also contribute to the final readout of the signal. The data presented here suggest that acylation of Hh proteins may provide another layer of complexity to this signal.

Acylation of Shh Has Varying Effects on Its Activity

Addition of an N-terminal acyl group to Shh has been shown to require the thiol group of Cys-24, the N-terminal residue of the mature signaling domain (Pepinsky *et al.*, 1998); it does not occur when Cys-24 is substituted by serine. However, this mutation does not affect the processing or secretion of Shh. Although C24S-Shh is much less active than wild-type Shh in ventralizing the embryonic mouse forebrain (Kohtz *et al.*, 2001), we show here that it retains significant limb patterning activity. It is possible that this reflects a greater sensitivity of the limb bud than the forebrain to Shh activity, allowing the limb bud to respond more strongly to the weakly active C24S-Shh. Alternatively, acylation could have tissue-specific effects on Shh function in mouse. Although the majority of Shh protein produced in tissue culture appears to be acylated (Pepinsky *et al.*, 1998), the distribution of acylated forms *in vivo* has not been evaluated. Acylation might be restricted to certain tissues, like the prechordal plate, or it might occur ubiquitously but be sensed specifically in the forebrain.

Although soluble and lipid-modified Shh bind the Ptc receptor with similar affinity in transfected EBNA-293 cells (Pepinsky *et al.*, 1998), and even deletion of 9 N-terminal residues of Shh-N does not affect Ptc binding in cell culture (Fuse *et al.*, 1999; Williams *et al.*, 1999), it is possible that tissue-specific modification of Ptc might allow it to specifically recognize acylated Shh. The N-terminal region of human Shh appears to form an extended structure protruding from the surface implicated in Ptc binding by modification studies (Fuse *et al.*, 1999; Pepinsky *et al.*, 2000). Alternatively, acylation of Shh might alter its affinity for other differentially expressed molecules involved in signaling or transport. These could be binding proteins such as Hip (Chuang and McMahon, 1999) that might preferentially bind unacylated Shh, reducing its effective concentration; factors required for extracellular transport, like the heparan sulfate proteoglycans synthesized by the product of the *tout velu* gene (Bellaiche *et al.*, 1998; The *et al.*, 1999); or hypothetical cofactors that would increase the activity of acylated Hh. The fibroblast cell line C3H10T1/2 appears to contain the acylation-sensitive determinants, as Shh without lipid modifications has a reduced effect on these cells

(Pepinsky *et al.,* 1998) and Shh-N missing amino acids 24–33 inhibits signaling to them by wild-type Shh-N (Katsuura *et al.,* 1999; Williams *et al.,* 1999). As studies in the mouse have so far been limited to ectopic expression of Shh, it would be of great interest to determine the effects of replacing wild-type Shh with a C24S mutant form.

Potential Roles of Acylation

Fatty acid chains are frequently added to intracellular proteins in order to localize them to the plasma membrane (Resh, 1999). Acylation of extracellular proteins has very rarely been reported, but it would be expected to reduce their solubility and restrict their diffusion. Hh has already been shown to carry a C-terminal cholesterol modification that restricts its action to nearby cells (Porter *et al.,* 1996a,b); misexpression of the mature N-terminal signaling domain without cholesterol increases its range of activity, resulting in patterning defects. A single lipid modification is usually insufficient for membrane localization (Resh, 1999); thus acylation and cholesterol modification could both be required to tether Hh molecules. However, we have no evidence that C24S-Shh and C84S-Hh are less efficiently localized than wild-type Shh or Hh, as antibody staining shows no difference in the distribution of the two forms when misexpressed (data not shown). Alternatively, the importance of acylation in addition to cholesterol modification could be to promote Hh association with raft membrane domains (Rietveld *et al.,* 1999); this may be important for its cellular trafficking or signaling.

If acylation were required to tether Hh molecules to the plasma membrane, increasing their local concentration, one might expect that events requiring short-range Hh signaling would be more sensitive than those that Hh can elicit at a longer range. However, it is not clear that the range of Hh action is the critical difference between vertebrate and fly limb patterning. There is some evidence that *Drosophila* Hh acts only as a short-range signal, as a large increase in the level of Hh expression in its normal domain has only minor effects (Fietz *et al.,* 1995). A long-range gradient model for Hh action would imply that such an increase would alter cell fates at a distance from the Hh domain to those normally induced by the higher levels of Hh closer to this domain. Although a gradient of Gli3 processing and widespread *Ptc* upregulation in the vertebrate limb bud have been taken as evidence that Shh acts as a long-range signal in limb patterning (Drossopoulou *et al.,* 2000; Wang *et al.,* 2000), membrane-tethered Shh can induce equivalent long-range transformations of digit identity (Yang *et al.,* 1997), suggesting that these may be due to a secondary signal.

Another possibility is that acylation might be required for Hh release from cells, perhaps localizing it to intracellular transport vesicles containing the Dispatched protein (Burke *et al.,* 1999). However, our finding that C84S-Hh can inhibit the function of wild-type Hh even when expressed only in the responding cells using *ptc*-GAL4 suggests that it is able to exit the cell, rather than just interfering intracellularly with the release of wild-type Hh. Similarly, the ability of C24S-Shh expressed in the limb ectoderm to activate *Hoxd13* expression in the underlying mesenchyme argues either that it can move freely between germ layers or that it activates a second signal with this ability. Thus it is likely that acylation is important for Hh signaling through Ptc, either because it causes more productive binding to Ptc itself or because it affects Hh interaction with other proteins.

C84S-Hh Antagonizes Wild-Type Hh Function

Our observation that overexpression of C84S-Hh has nonautonomous dominant negative effects on Hh function suggests that this mutant protein is able to compete with wild-type Hh for binding to a normal Hh target, but does not have signaling activity. As C84S-Hh affects the expression of the *dpp* and *ptc* genes and the Ci protein, which are known to be regulated by the Ptc receptor (Johnson *et al.,* 1995; Li *et al.,* 1995), this target is probably Ptc. Indeed, C24S-Shh-N is able to bind Ptc *in vitro* with equivalent or greater affinity than Shh-N (Williams *et al.,* 1999). This interpretation is also consistent with our observation that C84S-Hh has no dominant negative effect on *ptc* mutant clones. We do not know whether the C84S-Hh protein can be internalized on binding Ptc; although we see punctate Hh antibody staining in wing discs overexpressing C84S-Hh, its presence in the posterior as well as the anterior compartment and lack of consistent colocalization with punctate Ptc staining suggests that it could represent aggregation of the protein rather than internalization (J. D. Lee, data not shown). It is unlikely that C84S-Hh promotes the internalization of Ptc, since removal of Ptc from the cell surface appears to be sufficient for wild-type Hh function (Denef *et al.,* 2000).

It is possible that different target genes are differentially affected by mutations at the N-terminus of Hh. When C84S-Hh is expressed in the wing the 3–4 intervein region is deleted; this is precisely the region that requires direct Hh signaling rather than indirect patterning through Dpp. The serine–threonine kinase Fused and the COE protein Knot are both required specifically for the correct growth of the intervein region and the positioning of veins 3 and 4 (Busson *et al.,* 1988; Mohler *et al.,* 2000; Nestoras *et al.,* 1997; Vervoort *et al.,* 1999), and signaling through these proteins might be especially sensitive to Cys-84. However, the domain of dominant negative activity could also be attributed to a relatively weak inhibition of Hh function that can only be observed in regions that normally require the highest Hh concentrations (Mullor *et al.,* 1997; Strigini and Cohen, 1997). We note that overexpression of C24S-Shh in the posterior region of the mouse limb appears to have no inhibitory effect on endogenous Shh activity, suggesting that C24S-Shh cannot act as a Shh antagonist in this context, in spite of the antagonistic activity of C24S-Shh-N *in vitro* (Williams *et al.,* 1999). One possible difference

between these studies is the likely presence of cholesterol on the Shh molecules we used and not on the truncated domains produced for the *in vitro* study.

The Importance of the N-Terminal Cysteine Is Not Conserved

Our comparison of the effects of mutating the N-terminal residue of mature Hh in mouse and fly appendages reveals a surprising lack of conservation. Although Hh is required at the posterior of both the mouse limb and the fly wing, and may mediate some of its effects through BMP homologues in both cases, signaling requires an N-terminal cysteine only in *Drosophila.* Although Cys-84 is essential for the function of *Drosophila* Hh, mutation of this residue does not affect Hh processing (Fig. 3). The C84S-Hh protein also appears to be secreted as evidenced by its ability to alter wing patterning in the anterior compartment when expressed in either the posterior compartment or the anterior compartment and by its ability to nonautonomously lower the expression of Hh target genes. Acylation of the *Drosophila* Hh protein has not been directly demonstrated, due to the lack of sufficiently sensitive antibodies. However, acylation of human Shh was shown to occur more efficiently in insect cells than in mammalian cells in culture (Pepinsky *et al.*, 1998), indicating that the acylating enzymes are likely to be present in *Drosophila.* In addition, Kohtz *et al.* (2001) have shown that C24S-Shh and wild-type unacylated Shh produced in *E. coli* have equivalent activities on forebrain explants, suggesting that loss of acylation is the major effect of mutating this cysteine. However, it may not be the only effect, as C24S-Shh-N can antagonize the weak effect on C3H10T1/2 cells of unmodified Shh-N (Williams *et al.*, 1999). Thus Cys-84 could be directly recognized by downstream components in addition to its requirement for Hh acylation. It is also possible that *in vivo,* Cys-84 is modified by adducts other than fatty acids.

Because *Drosophila* cell membranes contain shorter phospholipids and are therefore fluid at lower temperatures than mammalian membranes (Rietveld *et al.* 1999), association with raft domains mediated by acylation could be more critical for *Drosophila* Hh. Alternatively, the differing importance of the N-terminal cysteine in flies and mice could simply indicate divergence of the protein components of both pathways. Indeed, we have found that misexpression of human Shh in the central domain of the *Drosophila* wing disc causes broadening of wing veins and differentiation of ectopic vein tissue, but not Hh-like expansions or duplications of the anterior compartment (J. D. Lee, unpublished data). This suggests that human Shh is not able to signal normally through *Drosophila* Ptc to induce *dpp* expression, although it could be causing ectopic vein formation by directly inducing the *vein* gene (Wessells *et al.*, 1999). The C24S mutant form of human Shh produces a weaker version of the same phenotype and has no apparent dominant negative effect on Hh signaling, again suggesting that it does not bind the same proteins as *Drosophila* Hh (J. D. Lee, unpublished data). This is surprising as zebrafish Shh has been shown to cause small anterior duplications in the fly wing when expressed surrounding the wing pouch (Ingham and Fietz, 1995); the difference could be due to the few sequence changes between zebrafish and human Shh or to the different domains of misexpression. The effects of protein modifications may be altered during evolution due to divergence of the interface between two interacting proteins. Our results suggest that modification of the N-terminal cysteine of Hh may contribute to altering its activity in a tissue- and species-specific manner.

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