Patched controls the Hedgehog gradient by endocytosis in a dynamin-dependent manner, but this internalization does not play a major role in signal transduction

Carlos Torroja, Nicole Gorfinkiel and Isabel Guerrero*

Centro de Biología Molecular ‘Severo Ochoa’, CSIC, Universidad Autónoma de Madrid, Cantoblanco, E-28049 Madrid, Spain

*Author for correspondence (e-mail: iguerrero@cbm.uam.es)

Summary

The Hedgehog (Hh) morphogenetic gradient controls multiple developmental patterning events in Drosophila and vertebrates. Patched (Ptc), the Hh receptor, restrains both Hh spreading and Hh signaling. We report how endocytosis regulates the concentration and activity of Hh in the wing imaginal disc. Our studies show that Ptc limits the Hh gradient by internalizing Hh through endosomes in a dynamin-dependent manner, and that both Hh and Ptc are targeted to lysosomal degradation. We also found that the ptc^14 mutant does not block Hh spreading, as it has a failure in endocytosis. However, this mutant protein is able to control the expression of Hh target genes as the wild-type protein, indicating that the internalization mediated by Ptc is not required for signal transduction. In addition, we noted that both in this mutant and in those not producing Ptc protein, Hh still occurred in the endocytic vesicles of Hh-receiving cells, suggesting the existence of a second, Ptc-independent, mechanism of Hh internalization.

Key words: Hedgehog signaling, Patched, Morphogenetic gradients, Dynamin, Deep Orange

Introduction

The formation of morphogenetic gradients is a recurring event during development. In Drosophila, the morphogens Wingless (Wg), Decapentaplegic (Dpp) and Hedgehog (Hh) are secreted from localized sources and determine, in a dose-dependent manner, the fate of cells several cell diameters away (reviewed by Vincent and Dubois, 2002). Three processes can affect the extension and slope of a morphogen gradient: the rate of production at the source, the rate of transport, and the rate of degradation. The mechanism by which these gradients form remains controversial. Yet despite theoretical and experimental studies supporting the idea that morphogen gradients can form by diffusion through the extracellular matrix (reviewed by Lander et al., 2002), it has been proposed that morphogens spread through repeated cycles of endocytosis and exocytosis, a process called transcytosis (Entchev et al., 2000; Greco et al., 2001). Furthermore, there is increasing evidence that endocytosis regulates the concentration and therefore the extension of morphogen activity (reviewed by Seto et al., 2002). It has been also recently proposed that self-enhanced ligand degradation underlies robustness of morphogen gradients (Eldar et al., 2003). Thus, Wg internalization and degradation modulate its asymmetric gradient in embryonic segments (Dubois et al., 2001) and also regulate the Wg gradient in wing imaginal discs (Strigini and Cohen, 2000). It is known that receptors play a central role in regulating endocytic trafficking. Hence, the morphogen receptor must also have a role in shaping the morphogen gradient. There are reports that altering the levels of the Dpp receptor, Thickvein, affects Dpp distribution (Lecuit and Cohen, 1998; Entchev et al., 2000).
regulate the Hh morphogenetic gradient (Tabata and Kornberg, 1994; Chen and Struhl, 1996; Martin et al., 2001; Strutt et al., 2001). Hitherto, it was believed that Hh effectively promoted its own sequestration (reviewed by Ingham and McMahon, 2001) by upregulating ptc transcription. This negative feedback mechanism restrains the range of Hh signaling. However, the cellular mechanisms by which Ptc controls the Hh gradient remain unclear.

We investigated the role of Ptc-mediated endocytosis in the control of Hh gradient formation and signaling in the A compartment of the wing imaginal disc. Our data show that Ptc limits the Hh gradient by internalizing Hh through endosomes in a dynamin-dependent manner and that this Hh-Ptc complex is targeted to degradation by lysosomes. We have genetically uncoupled the two proposed functions of Ptc, through the analysis of the ptc\textsuperscript{14} mutant that is unable to sequester Hh and yet retains a normal capacity to mediate Hh signaling. Our analysis leads to the suggestion that Hh signaling can occur in the absence of Ptc-mediated Hh internalization.

Materials and methods

Fly stocks

Description of mutations, insertions and transgenes in FlyBase are available at http://flybase.geneontology.org.

Chromosomes used were as follows.

\begin{align*}
\text{FRT42D} \\
\text{P [ry+7.2]=neoFRT/42D arm-lacZ} \\
\text{P [ry+7.2]=neoFRT/42D P[ubi-GFP(S65T)nlsls]} \\
\text{FRT18A} \\
\text{w[1118] P[ubi-GFP(S65T)nlsls] P[neoFRT/18A]} \\
\text{w[1118] arm-lacZ P[neoFRT/18A]} \\
\text{Flipase} \\
\text{Hsp70-flipase chromosomes were provided by G.Struhl.}
\end{align*}

The amorphic ptc\textsuperscript{14} allele, the embryonic lethal alleles ptc\textsuperscript{12} and ptc\textsuperscript{14} (also known as ptc\textsuperscript{1087}) were from Tübingen (Nüsenlin-Volhard and Wieschaus, 1980).

In addition, the null dor\textsuperscript{1} allele (Shestopal et al., 1997) and the temperature-sensitive, shi\textsuperscript{111} (Grigliatti et al., 1973) and hh\textsuperscript{12} (Ma et al., 1993) alleles were used. The restrictive temperatures for the shi\textsuperscript{111} and hh\textsuperscript{12} alleles are 32°C and 29°C, respectively.

The reporter genes used were dpp-LacZ\textsuperscript{1.0}, expressed as the endogenous RNA in the imaginal disc (Blackman et al., 1991); dpp\textsuperscript{10638} (a dpp mutant with a lacZ insertion) (Zecca et al., 1995); and ptc-lacZ (a lacZ insertion in the ptc gene), which was a gift from C. Goodman.

The Gal4 drivers for ectopic expression experiments using the Gal4/UAS system (Brand and Perrimon, 1993) were the following: c765-Gal4 (ubiquitously expressed in the wing disc (Nellen et al., 1996)), ptc-Gal4 (a gift from J. Campos-Ortega), hh-Gal4 (a gift from T. Tabata) and AB1-Gal4 (Munro and Freeman, 2000).

Genotypes of larvae for generating mosaic clones

Mutant clones

Clones were generated by FLP-mediated mitotic recombination. Larvae of the corresponding genotypes were incubated at 37°C for 1 hour at 24-48 hours after egg laying (AEL), or for 45 minutes at 48-72 hours AEL. The genotypes of the flies for clone induction were:

\begin{itemize}
\item y, w, FLP/++; en-Gal4 / UAS-HhGFP, FRT82 arm-lacZ / FRT82B disp\textsuperscript{2037707}.
\item shi\textsuperscript{111} FRT18A / arm-lacZ FRT18A; FLP/+
\item FRT42D ptc\textsuperscript{12} / FRT42D ubi-GFP; FLP/+
\item FRT42D ptc\textsuperscript{14} / FRT42D ubi-GFP; FLP/+
\item dor\textsuperscript{1} FRT18A / arm-lacZ FRT18A; FLP/+
\item dor\textsuperscript{1} FRT18A / arm-lacZ FRT18A; FLP/+
\item FRT18A / arm-lacZ FRT18A; FLP/+
\end{itemize}

\textbf{Transgenes}

Hh transgene

For the HhGFP fusion protein, the GFP-coding sequence was amplified by PCR from a pEGFP-N1 vector (Clontech, catalog number 6085-1) and tagged in frame before cleavage site of Hh by auto-proteolysis (…SH254-GFP-V255HGCF…) (Fig. 1A).

Ptc transgenes

To obtain the Ptc\textsuperscript{14} mutation (L83Q), the QuickChange\textsuperscript{TM} Site-Directed Mutagenesis Kit (Stratagene) was used to introduce a point mutation into the ptc cDNA (T242A). This was confirmed by sequence analysis. The GFP-tagged proteins of ptc\textsuperscript{12}, ptc\textsuperscript{14} and ptc\textsuperscript{14} were cDNA fusions cloned in frame by PCR into a pEGFP-N1 vector (Clontech, catalog number 6085-1). The GFP sequences were tagged in frame in the C-terminal regions of both Ptc\textsuperscript{12} and Ptc\textsuperscript{14}. The stop codon was substituted by a glycine (codon 1287), followed by a valine (codon 1288) and the first methionine of the GFP sequence (Fig. 2A). ptc-GFP fusions were transferred to the pUAS vector. Several transgenic lines were obtained. Experiments involving the three Ptc protein forms (wild-type and mutant forms) were conducted using the transgenic fly lines that showed similar levels of Ptc expression after induction with the same Gal4 line.

Other transgenic flies

\textbf{Functional characterization of HhGFP fusion protein}

It was expected that the fusion of the GFP sequence to Hh cDNA at position between codons 254 (H) and 255 (V) did not interfere neither with the processing, secretion and diffusion process of Hh nor with the Hh binding to Ptc (Burke et al., 1999; Pepinsky et al., 2000). To determine whether the auto-proteolysis of HhGFP occurs normally, we analyzed the presence of the distinct species of Hh by western blot using either anti-Hh antibody as it was previously reported (Lee et al., 1994; Tabata and Kornberg, 1994; Porter et al., 1995). Three major bands were also observed in salivary glands extracts from ptc\textsuperscript{14}\textsuperscript{1}HhGFP flies, three bands of ~45 kDa (U in Fig. 1B, row 1; uncleaved Hh), 25 kDa (C in Fig. 1B, row 1; C-terminal half of Hh) and 20 kDa (N in Fig. 1B, row 1; N-terminal active form) were observed using anti-Hh antibody as it was previously reported (Lee et al., 1994; Tabata and Kornberg, 1994; Porter et al., 1995). Three major bands were also observed in salivary glands extracts from ptc\textsuperscript{14}\textsuperscript{1}HhGFP flies using either anti-Hh or anti-GFP antibodies. The molecular weight of these bands corresponded to the ones expected for the un-cleaved HhGFP chimera (70 kDa, U in Fig. 1B,
Role of Patched in Hedgehog signaling

Fig. 1. The HhGFP chimera behaves as the wild-type Hh protein. (A) Scheme of the predicted HhGFP fusion protein processing. HhGFP-F, full-length protein; HhGFP-U, the unprocessed protein without the signal peptide; HhC, the C-terminal region of the processed protein; HhGFP-Np, the N-terminal region of the processed protein with the GFP fragment and the palmitic acid and cholesterol modifications. (B) Western blot of protein extracts from AB1-Gal4/UAS-Hh and AB1-Gal4/UAS-HhGFP salivary glands stained with anti-Hh or anti-GFP. U, unprocessed protein (HhU); N, processed protein (HhNp); C, catalytic fragment (HhC). (C) Ectopic clones of HhGFP (green) in A cells (labeled by β-Gal staining, blue) of a wing imaginal disc stained with anti-En (red). Ectopic expression of HhGFP in A wing pouch cells is able to induce En expression inside the clone and non-autonomously in a region of three to four cell diameters around the clone (arrowheads in red panel) as wild-type Hh. Note that HhGFP vesicles are detected in cells far from the source (arrows in green panel). (D) hh-Gal4/UAS-HhGFP wing imaginal disc stained with anti-Ptc (red). (d) Magnification of the boxed area in D. HhGFP signal is observed in A cells co-localizing with Ptc protein in vesicles (arrowheads). (E) disp– clones (labeled by the lack of β-gal staining, red) in an en-Gal4/UAS-HhGFP (green) wing imaginal disc. There is an accumulation of Hh-GFP in the disp– mutant cells (arrowheads) with respect to the wild-type region (asterisk). (F) en-Gal4/UAS-HhGFP; hh ts2 mutant flies raised at the restricted temperature during larvae development. All the structures were rescued except for some head structures (arrow) where the expression of en depends on Hh expression. In this and all other figures, the A compartment of the wing disc is towards the left and the P is towards the right. A broken line indicates the AP compartment border. Scale bars: 15 μm in C,d; 50 μm in D,E.

 rows 2 and 3), for the HhGFP-Np (46 kDa, N in Fig. 1B, rows 2 and 3) and for the HhC (25 kDa, C in Fig. 1B, row 2), indicating that the Hh-GFP processing occurs normally.

To assay if HhGFP is secreted and spread normally we analyzed the effect of ectopic HhGFP expressing clones in the wing imaginal disc. In the A compartment of the wing pouch, these clones were able to induce En expression autonomously and non-autonomously (Fig. 1C) as do the wild-type Hh protein (Basler and Struhl, 1994; Zecca et al., 1995). Co-localization of Ptc and HhGFP in vesicles was observed in the A cells close to the AP border in an hh-Gal4/UAS-HhGFP wing disc (Fig. 1D,d).

To test if HhGFP was modified by cholesterol we induced disp– (disp–) mutant clones in an en-Gal4/UAS-HhGFP wing disc. It has been reported a membrane accumulation of Hh-Np in the absence of Disp that is dependent on its cholesterol modification (Burke et al., 1999). As it was expected, disp– clones induced in the P compartment showed an autonomous accumulation of Hh-GFP compared with the wild-type territory (Fig. 1E). This result indicates that HhGFP-Np is cholesterol modified.

Finally, to determine if HhGFP can substitute the endogenous function of Hh, we performed rescue experiments of the hh mutant phenotype. hh2– flies raised at the restricted temperature (29°C) for the same developmental period were rescued to pharates showing normalized notum, legs, abdomen and genitalia (Fig. 1F). The wings were present but not extended. In the head, some structures were rescued but the dorsal head, the ocelli and the eyes were not rescued probably because en expression begins after the onset of hh expression (Royet and Finkelstein, 1997). These results indicate that HhGFP is able to substitute the wild-type Hh function.

Functional characterization of PtcGFP fusion proteins

To characterize the PtcGFP fusion proteins first, we analyzed the integrity of the fusion proteins by western blot. We detected the presence of a protein band of 180 to 200 kDa approximately in extracts of AB1-Gal4/UAS-PtcWT GFP or UAS-Ptc14GFP salivary glands using the anti-GFP antibody (Fig. 2B). This band has a molecular weight higher than the expected 16,964 kDa based on DNA sequence, probably because Ptc has six potential glycosylation sites.

Then, we assayed the ability of the Ptc WT GFP fusion protein to sequester Hh when it was expressed in the P compartment. Ectopic expression of the wild-type Ptc in the P compartment reduces the range of Hh signaling (Johnson et al., 1995) and induces the accumulation of Hh (Martín et al., 2001). The expression of Collier, one of the Hh targets, was reduced when PtcWT GFP was overexpressed in the P compartment (Fig. 2D). In addition, ectopic
PtcWTGFP clones in the P compartment induced an autonomous accumulation of Hh (see Fig. 7A). Finally, we observed a downregulation of Smo in PtcWTGFP ectopic clones in the P compartment (Fig. 2E) as it has been reported for the wild-type Ptc (Denef et al., 2000; Martin et al., 2001). Collectively, these data indicate that PtcWTGFP behaves as the wild-type protein.

Immunostaining of imaginal discs and western blot analysis

Immunostaining was performed following standard dilutions: rabbit polyclonal anti-Hh 1/400 (raised for the present study), rat polyclonal anti-Hh 1/100 (Sánchez-Herrero et al., 1996), mouse monoclonal anti-Ptc (Apa 1.3) (Capdevila et al., 1994b) 1/100, rabbit polyclonal anti-β-gal (Jackson Laboratories) 1/1000, rabbit polyclonal anti-GFP (Molecular Probes, catalog number A-6455) 1/100, rabbit polyclonal anti-Col antibody (Vervoort et al., 1999) 1/200, mouse monoclonal anti-En (Patel et al., 1989) 1/10, mouse monoclonal anti-Wg (Brook and Cohen, 1996) 1/50, rat polyclonal anti-Smo (Denef et al., 2000) 1/200, rat monoclonal anti-Ci (Motzny and Holmgren, 1995) 1/10 and rat polyclonal anti-Caupolican (Diez del Corral et al., 1999) 1/1000. Stained imaginal discs were examined using a BioRad confocal laser-scanning microscope.

For western blot analysis, protein extracts from salivary glands of AB1-Gal4/UAS-PtcWTGFP or AB1-Gal4/UAS-Ptc14GFP flies were prepared in Laemmli buffer and resolved by SDS-PAGE, immunoblotted and then analyzed using anti-Hh (1/500) (Tabata and Kornberg, 1994) and anti-GFP (1/1000) (Molecular Probes, catalog number A-6455) antibodies. Horseradish peroxidase-conjugated secondary antibodies were used to develop the signal using the ECL Western Blotting Analysis System (Amersham Pharmacia).

Labeling the endocytic compartment

Third instar larval discs were incubated in 3.7 mM Red-dextran (lysine fixable, MW3000, Molecular Probes) in M3 medium at 25°C (pulse) and then washed five times for 2 minutes in ice-cold M3 medium. The discs were then incubated for a chase period at 25°C in M3 medium prior to fixation in 4% paraformaldehyde. To visualize the early endosomes of the endocytic compartments, the discs were pulsed for 5 minutes without a chase period. For late endosomes, a 5 minutes pulse and 60 minutes chase were used. This was followed by fixing in PBS 4% paraformaldehyde (PF) for 40 minutes at 4°C and in 4% paraformaldehyde in PBS 0.05% Triton X-100 for 20 minutes at room temperature. The discs were then washed and incubated with antibodies in PBS 0.05% Triton X-100.

Extracellular labeling of HhGFP in shi-ts1 mutant clones

This protocol was modified from that described by Strigini and Cohen (Strigini and Cohen, 2000). Larvae containing shi mutant clones where maintained at restrictive temperature (32°C) for 3 hours, dissected at restrictive temperature and transferred immediately to ice-cold M3 medium containing anti-GFP antibody. They were then incubated at 4°C for 30 minutes, washed in ice-cold PBS and fixed in PBS 4% PF at 4°C. By transferring the discs from 32°C to ice-cold medium, the cells stop their vesicular trafficking and the proteins remain at their subcellular locations at the moment of cooling. Incubating with the anti-GFP antibody under these ‘in vivo’ conditions, without detergents and prior to fixation, rendered the antibody incapable of penetrating the cells. Thus, only extracellular antigen was labeled. The antibody does not label intra-cellular HhGFP. The lack of staining of the intracellular HhGFP vesicles in the P compartment of the same disc (see Fig. 3b, arrows) was used as a control for this specific extracellular anti-GFP antibody staining method.

Extracellular labeling of Ptc in ectopic PtcWTGFP and Ptc14GFP clones

Larvae containing ectopic PtcWTGFP and Ptc14GFP clones were dissected and incubated in ice-cold medium with anti-Ptc antibody at 1/10 dilution in M3 medium for 30 minutes, washed with ice-cold PBS and fixed with 4% paraformaldehyde for 20 minutes at 4°C and 20 minutes at 25°C. After fixation, discs were incubated with the corresponding secondary antibody.

Quantification analysis

To quantify the rate of endocytosis of PtcWT and Ptc14 proteins, ectopic PtcWTGFP and Ptc14GFP clones in the A compartment were induced and the endocytic compartment was labeled by incubation with Red-dextran (10 minute pulse). Using Metamorphic software, the PtcGFP fluorescence in the Red-dextran vesicles was measured and compared with the total PtcGFP signal in the clone. The resulting ratio was normalized to the Red-dextran vesicles in the clone cells. For this analysis, six confocal sections of seven different clones were measured and a total of 1459 vesicles for Ptc14 and 1650 for PtcWT were analyzed.

The content of HhGFP in the dotted pattern in wild-type and ptc16 mutant clones was also measured with Metamorph analysis software.
Each clone analyzed was compared with an equivalent region in the wild-type territory of the same wing disc. The result from five different clones from independent experiments shows a ratio between 4 and 5 times more Hh in the wild-type cells.

**Results**

**Patched controls the endocytosis of Hedgehog in a dynamin-dependent manner**

In the wing imaginal disc epithelium, the A cells adjacent to the AP border express high levels of Ptc protein, the only known Hh receptor that mediates Hh endocytosis in *Drosophila* (Martín et al., 2001). To better analyze the Hh and Ptc interaction, we have used both HhGFP and PtcGFP tagged proteins in some of the experiments presented in this work. These fusion proteins behave as wild-type ones (for details, see Materials and methods, Figs 1, 2).

Here, we asked whether Hh internalization was required for the formation of the Hh gradient. In other signaling mechanisms, such as that of Dpp, the internalization of the morphogen is required for gradient formation (Entchev et al., 2000; Teleman and Cohen, 2000). To test this hypothesis, we used a thermo-sensitive *shibire* (*shi*) mutation. Shibire, the *Drosophila* Dynamin homolog, is needed for fission of clathrin-coated vesicles and the internalization of caveolae (van der Bliek, 1999). Shi mediates Ptc internalization in *Drosophila* embryos. In *shi* mutant embryos, a punctuate accumulation of Ptc at the plasma membrane was observed (Capdevila et al., 1994b), probably as a consequence of the accumulation of ‘coated pits’ at the plasma membrane (Kosaka and Ikeda, 1983). Here, we also observed a punctate accumulation of both Ptc and Hh at cell surface in *shits1* clones, close to the AP compartment border of the wing imaginal disc (Fig. 3A). To test if the Hh accumulation was extracellular we used HhGFP fusion protein. *Hh-Gal4/UAS-HhGFP* third instar larvae containing *shis1* clones were maintained at 32°C for 3 hours, a temperature that blocks Shi-dependent trafficking. Discs were then dissected and incubated with anti-GFP antibody in ice-cold medium prior to fixation. Under these experimental conditions, all trafficking processes were blocked and the antibody only labeled extracellular HhGFP molecules, while GFP fluorescence labeled both the intracellular and extracellular Hh. We noted that in the *shi* clone, all the green (HhGFP) fluorescence colocalized with the red (antibody-conjugated) fluorescence, indicating that all the retained Hh was extracellular (Fig. 3B,b, arrowheads).

Next, we addressed the question of whether Ptc might be responsible for the accumulation of Hh at the cell surface when endocytosis was blocked. To achieve this we made *ptc16* clones (an amorphic allele, which does not produce Ptc protein, also known as *ptcIIW109*) (Capdevila et al., 1994a) in a *shis1* background. We found no build-up of Hh when Ptc
Fig. 4. Hh signaling is unaffected in dor mutant cells. (A) A confocal section of a Rab-7GFP/c765-Gal4 wing imaginal disc, stained with anti-Ptc (blue) and anti-Hh (red) antibodies. (a) Magnification of boxed area in A. The staining shows co-localization of Hh, Ptc and Rab-7 at the AP compartment border (circles in a). There are also spots of Ptc and Hh colocalization that do not colocalize with Rab7 (arrowheads in a); these are probably early endosomes. The inset in a shows detail of the co-localization of the three proteins at some of these spots. Rab-7 shows doughnut-shaped GFP emission (arrow) surrounding the Ptc and Hh spots (arrowheads). (B) dor mutant clones (labeled by the lack of β-gal staining, blue) at the AP compartment border showing high accumulation of Hh (green) and Ptc (red) proteins because of the inhibition of lysosomal degradation. (C) Plot showing the green color pixel intensity of the cells in clone 1 (blue line) and clone 2 (red line) corresponding to boxed areas in B (green panel) with respect to the distance of the cells to the AP border. The same intensity (Hh accumulation) is seen in the A cells located at the same distance from the AP compartment border in A and B. Moreover, we did not observe an increase in the distance that Hh moves or signals in the anterior side of the shits1 clone (Fig. 3D, arrowhead). These observations suggest that although internalization of Hh is blocked in the shits1 mutants, Ptc has already sequestered Hh. This interpretation is in agreement with the blockage in Dynamin function at a stage where commitment to endocytic sequestration has already occurred (Kosaka and Ikeda, 1983; Ramaswami et al., 1994; Guha et al., 2003).

After internalization, Ptc and Hh are targeted to degradation

We previously reported that Ptc moves Hh to the endocytic compartment in the wing imaginal disc (Martín et al., 2001), after which degradation of Hh and Ptc would be expected. Rab-7 GTPase controls both protein trafficking to late endosomes and their subsequent fusion with lysosomes (reviewed by Zerial and McBride, 2001). A Rab-7GFP (Entchev et al., 2000) was used for labeling late endosomes. Ptc-Hh vesicles were found to colocalize with Rab-7GFP when this marker was expressed in all wing imaginal disc cells (Fig. 4A,a, circles). These results suggest that after internalization, Ptc and Hh go to late endosomes and lysosomes. Next, we examined whether the targeting of Hh to the degradative lysosomal pathway had any effect on Hh spreading and signaling. To block the degradative pathway, we used deep orange (dor) mutants, one of the mutations that affects eye pigmentation in Drosophila and is required for normal delivery of proteins to lysosomes (Sevrioukov et al., 1999). dor encodes the homolog of the yeast vacuole sorting protein Vps18p, a protein that as part of a complex regulates the function of Ypt7p, the paralog of Rab-7 in yeast (Rieder and Emr, 1997; Price et al., 2000). Blockage of the degradative pathway in dor mutant clones resulted in the accumulation of both Hh and Ptc (Fig. 4B), which was not observed when wild-type discs were stained with anti-Hh antibodies. As we have observed in shits1, despite the large accumulation of Hh and Ptc in dor clones, the activation of Hh target genes remains unchanged, as shown by the normal dpp-lacZ (compare mutant territory in Fig. 4D, arrowhead, with wild-type territory, arrow) or ptc-lacZ expression (data not shown). These results indicate that both spreading and signaling are normal when Hh degradation is blocked in dor cells. Furthermore, the intensity of Hh immunofluorescence in the A cells located at the same distance from the AP border remains the same (Fig. 4B,C arrowheads), regardless of whether Hh had crossed wild-type (clone 1) or mutant (clone 2) territory. These data indicate that Ptc levels control the Hh gradient by sequestering Hh, and then both Hh and Ptc are targeted to degradation. They also
suggest that Hh signaling is independent of Hh degradation by lysosomes.

**Genetic uncoupling of the two Ptc functions**

The analysis of Hh signaling in *shi* and *dor* mutant clones suggested that Hh sequestration and Hh signaling are independent. It has been proposed that Ptc regulates both signaling and sequestration (Chen and Struhl, 1996). This hypothesis was based on the analysis of the *ptc* S2 allele, which affects Hh signaling, but the sequestration of Hh is normal (Chen and Struhl, 1996; Martín et al., 2001; Strutt et al., 2001). The PtcS2 mutant protein has a point mutation in the sterol-sensing domain (D584N) (Martín et al., 2001; Strutt et al., 2001) showing a preferential accumulation in endosomes (Martín et al., 2001).

As Ptc S2 is defective only in its signaling function, we used the *ptc* S2 allele to test for complementing alleles of *ptc* that might be defective for sequestration but not signaling. In this way, we identified the embryonic lethal *ptc* 14 allele (also known as *ptc IIR87*). Homozygous somatic *ptc* 14 mutant clones induced in the A compartment produce the described *ptc* mutant phenotype caused by the full activation of Hh targets (Phillips et al., 1990; Capdevila et al., 1994a; Tabata and Kornberg, 1994). By contrast, *ptc* 14 mutant clones did not activate Hh signaling in the A compartment outside the AP border, as shown by the expression of Hh target genes such as *en, col, dpp, iro* or *ptc* itself (Fig. 5B-F clones 1, 3, 5; data not shown). Large clones (24-48 hours AEL) abutting the AP border activated En (Fig. 5B, clone 2), Col (Fig. 5D, clone 4) and *dpp* (Fig. 5F, clone 6) outside their normal expression domains. In addition to this activation, short-range, non-autonomous activation of En, Col and Dpp extended a few cells outside the clone (arrowheads). To determine whether this effect was due to the presence of Hh, *ptc* 14 clones were induced in an *hh* ts2 mutant background. After 30 hours at the restrictive temperature, *ptc* 14 clones located neither outside nor close to the AP border activated Hh targets genes (Fig. 6B,D). These results indicate that Ptc14 activates the Hh pathway only in the presence of Hh, similar to wild-type Ptc. Furthermore, the broadened activation of target genes outside their normal expression domains in clones abutting the AP border indicates that *ptc* 14 cells do not sequester Hh efficiently.
described as a null allele based on its embryonic phenotype (Nusslein-Volhard and Wieschaus, 1980) (Fig. 6E,F, middle panels). Nevertheless, the embryonic phenotype of the double mutant ptc14; hhts2 at the restrictive temperature (Fig. 6E, right panel) resembled that of hh embryos, with no Wg expression (Fig. 6F, right panel). This indicates that the extended Wg expression observed in ptc14 mutant embryos was due to the presence of Hh, and not to the constitutive activation of the Hh pathway caused by the loss of ptc function.

Endocytosis defect in the Ptc14 mutant

The extended gradient of Hh across ptc14 mutant cells could be due to a defect in Ptc-Hh sequestration. The sequence of this allele confirmed a leucine to glutamic acid change (L83Q) at the first trans-membrane domain as previously reported (Strutt et al., 2001). To compare the ability of Ptc14 to sequester Hh with that of wild-type Ptc, both mutant and wild-type proteins were C-terminally tagged with GFP. Using these GFP tagged Ptc proteins we observed that the accumulation of Hh was much lower in Ptc14GFP ectopic clones than in PtcWTGFP ectopic clones in the P compartment (compare Fig. 7A,a with 7B,b). This implies that Ptc14 sequesters Hh quite inefficiently.

Next, we analyzed the internalization of Hh and Ptc in ptc14 clones abutting at the AP compartment border. Fig. 7C shows a wing disc containing a ptc14 clone and its wild-type twin clone. The wild type AP border cells show the normal pattern of Hh and Ptc accumulation in endocytic vesicles (circles in Fig. 7c). In the ptc14 mutant clone, Ptc staining showed the same protein levels as in the wild-type territory (Fig. 7C, green panel). However, Ptc14 was not confined to punctuate structures, and Hh did not co-localize with Ptc14 (compare Fig. 7c and 7c’). This observation is in agreement with the lack of Hh sequestration observed in the ectopic Ptc14 mutant clones induced in the P compartment (Fig. 7B). To analyze this sequestration defect further, we made clones of ptc14 in a shi61 background and found that Hh was not accumulated at the plasma membrane inside the clone (Fig. 7D, asterisk) while in the Ptc wild-type cells anterior to the clone Hh was accumulated (Fig. 7D, arrowhead). This accumulation indicates that Hh can spread further through a ptc14 mutant territory than a wild-type territory. The behavior of Hh in these shi61; ptc14 double mutant cells was similar to that observed in ptc16 clones (without Ptc protein) in a shi61 background (Fig. 3C). This result indicates that Ptc14 does not sequester Hh properly.

To explore a most likely failure in Ptc14-mediated endocytosis of Hh, we examined the presence of Ptc14 compared with wild-type Ptc protein in endosomes. In wild-type cells, Ptc accumulates in early endocytic vesicles (Fig. 8A,a, circles), while in ptc14 cells Ptc14 does not accumulate in these structures (Fig. 8B,b, arrowheads). This lack of colocalization of Ptc14 with the endosomal marker could be due to the absence of Ptc14 at the plasma membrane. To test this possibility, discs expressing Ptc14GFP ectopic clones were incubated ‘in vivo’ with an anti-Ptc antibody raised against the first extracellular loop of the Ptc protein (Capdevila et al., 1994b), in conditions of blocked trafficking processes (see Materials and methods). The extracellular labeling of Ptc in both PtcWTGFP (Fig. 8C) and Ptc14GFP (Fig. 8D) ectopic clones indicates the presence of the mutant protein at the plasma membrane.

Having demonstrated that Ptc14 reaches the plasma membrane, we tested if the reduced ability of Ptc14 to internalize Hh was due to a defect of Ptc14 itself to enter the endocytic compartment. Thus, we examined the competence of ectopic PtcWTGFP and Ptc14GFP to produce endocytic vesicles either in the presence or in the absence of Hh. In the absence of Hh, wild-type Ptc protein also accumulated in endosomes, as shown in PtcWTGFP ectopic clones located in areas of the A compartment that did not receive Hh (Fig. 8E,e, circles). This indicates that Ptc protein moves from the plasma membrane to the endocytic compartment in a ligand-independent manner, as has been shown in vertebrate cells (Incardona et al., 2002). However, Ptc14GFP ectopic clones showed reduced amount of Ptc in endosomes, either in the presence (Fig. 8B,b, arrowheads) or absence of Hh (Fig. 8F,f, arrowheads). Therefore, we can conclude that Ptc14 cannot internalize Hh because of defective endocytosis by Ptc14.
The two functions of Ptc take place in different subcellular compartments

The complementation of ptc14 and ptcS2 alleles clearly indicates that Ptc has separate functions (one involved in signaling through an interaction with Smo and the other in Hh sequestration). But how Ptc performs both functions remains controversial. One can envision that if both proteins are localized in different subcellular compartments, indirect complementation might occur. Thus, while PtcS2 is sequestering Hh in endosomes, Ptc14 could be interacting with Smo to control Hh signaling. Alternatively, both proteins PtcS2 and Ptc14 might form a complex as part of the Ptc receptor, as previous studies have suggested (Johnson et al., 2000; Martín et al., 2001). To discriminate between these two possibilities, we used tagged versions of PtcWTGFP, PtcS2GFP and Ptc14GFP to analyze the subcellular distribution of these mutant proteins in imaginal disk (data not shown) and salivary glands cells. Salivary glands cells are convenient for these experiments because are large and express endogenous Ptc protein at very low levels when Hh is not present (Zhu et al., 2003). In both systems, both PtcWTGFP and PtcS2GFP proteins were localized in large vesicles (Fig. 9A, B). However, Ptc14GFP was found at the plasma membrane, consistent with the observations made in ptc14 mutant clones, and also in the membrane of the secretion vacuoles (Fig. 9C). Next, we examined the sub-cellular distribution of Ptc14GFP protein when co-expressed with PtcS2 (without GFP). Under these conditions, Ptc14GFP was localized in vesicles resembling to PtcWTGFP localization (Fig. 9D). This change in the subcellular distribution of Ptc14GFP strongly suggests that PtcS2 and Ptc14 form a complex that re-establishes the wild-type function. Furthermore, these observations indicate that Hh signaling and sequestration take place in different subcellular compartments.

Is Hh still internalized in the absence of Ptc?

As Hh even signals normally in ptc14 mutant cells without Ptc mediated internalization, we went on to explore whether the mutant cells still internalized some Hh. So, we compared the internalization of Hh in either ptc14 clones or in clones of the null allele ptc16 induced at the AP compartment border in Hh-Gal4/HhGFP imaginal discs. In both cases, the same low amount of Hh was found in vesicles that did not co-localize with Ptc (Fig. 10A, a, C, arrowheads), but colocalize with internalized Red-dextran (Fig. 10B, b, D, d, circles). If Ptc were the only receptor for Hh, we would not expect to find Hh inside ptc16 cells, suggesting the presence of a Ptc-independent Hh internalization mechanism in the A compartment.

Discussion

We report genetic studies on Ptc function and its role in endocytosis during Hh signaling in Drosophila. We are able to show through the analysis of ptc14, a mutant that does not internalize Hh but is able to perform Hh signal transduction, that both proposed Ptc functions are genetically uncoupled. We also show that Ptc limits the Hh gradient by internalizing Hh in a dynamin-dependent manner, and that this Hh-Ptc complex is targeted to the degradation pathway. These findings strongly suggest that internalization mediated by Ptc shapes the Hh gradient and also lead to the challenging suggestion that Hh signaling can occur in the absence of Ptc-mediated Hh.
internalization. We will now discuss the two functions of Ptc in Hh signal transduction in the light of our results.

**Role of Ptc in Hh gradient formation**

We have shown here that Hh and Ptc sorting to the endocytic membrane-bound compartment plays a crucial role in modulating Hh levels during development. A strong support of the conclusions in this work comes from the analysis of the ptc14 allele. Although ptc14 mutants are lethal with a strong ptc–embryonic phenotype, Ptc14 mutant cells in the imaginal discs showed an effect only when the clone touched the AP compartment border but not in any other part of the disc. This result indicates that the presence of Hh is required to reveal a defect in Ptc14 function. This Hh requirement has been probed by the lack of activation of the Hh targets in ptc14 cells in the absence of Hh either in the embryos or in the imaginal discs. The complementation of ptc14 with ptcS2 allele, which is considered as null for blocking Hh signal transduction and acts as dominant negative (Martín et al., 2001; Strutt et al., 2001), indicates that Ptc14 does not have a greater sensitivity to Hh than the Ptc wild-type protein. Conversely, it is shown here that there is a decrease of internalization of Hh in ptc14 mutant clones compared with wild-type Ptc territory and an extension of the range of Hh gradient. Therefore, we can conclude that Ptc14 is unable to sequester Hh efficiently in either the embryo or imaginal discs and that the ptc14 embryonic phenotype would be the result of greater spreading of Hh and not to the constitutive activation of the Hh pathway.

Ptc14 responds to Hh as the wild-type Ptc protein and activates the signaling pathway indicating that the interaction of Ptc14 and Hh is probably normal. However, this Hh-Ptc interaction does not necessarily imply sequestration. Although Ptc14 occurs at the plasma membrane, we do not observe internalization of Hh or extracellular Hh accumulation in ptc14 mutant clones. These results, therefore, suggest that Hh-Ptc interaction is not sufficient to sequester Hh and that an active internalization process of Hh mediated by Ptc is required. This Hh internalization mediated by Ptc is Dynamin-dependent, based on the membrane accumulation of Hh and Ptc in shi mutant clones and the lack of accumulation of Hh in shi ts1; ptc16 double mutant clones. However, the initiation of the internalization process is not blocked in shi mutants because Dynamin is required for fission of clathrin-coated vesicles after the internalization process has already started (Kosaka and Ikeda, 1983; Ramaswami et al., 1994; Guha et al., 2003). This fact would explain why Hh gradient and signaling is not extended when endocytosis is blocked in shi mutant cells. As Ptc14 seems to have a problem in entering the endocytic compartment and we
have not found Hh accumulation in shi"ts1; ptc"14 double mutant clones we conclude that the initiation of the internalization process does not occur in Ptc"14. Taken together, these data indicate that only when Ptc forces Hh to the endocytic pathway Hh is sequestered in the receiving cells.

The behavior of Hh and Ptc in dor" cells indicates that after sequestration, Ptc internalizes Hh, and both Hh and Ptc are degraded. Thus, controlling both endocytosis and degradation of Hh modulates its gradient. Similar mechanisms have been described for controlling the asymmetric gradient of Wg in embryonic segments (Dubois et al., 2001). It is possible that additional factors may contribute to shaping the Hh gradient, because in large ptc" clones close to the AP border, which lack Ptc protein to sequester Hh, an Hh gradient in endocytic vesicles was also observed, although the range of this gradient was more extended than in wild-type cells (data not shown). This is consistent with two mechanisms of Hh internalization in Hh receiving cells as we have observed, one mediated by Ptc and another not mediated by Ptc.

**Role of Ptc in Hh signal transduction**

From studies in both vertebrates and Drosophila, it was thought that Hh protein binds to Ptc (Stone et al., 1996; Fuse et al., 1999). Ptc is then internalized and traffics Hh to endosomal compartments where both are degraded (Denef et al., 2000; Incardona et al., 2002), the entire process triggering activation of the Hh pathway. It is shown here that Ptc"14 responds to Hh as would the wild-type Ptc protein in activating the pathway.
However, Ptc\textsuperscript{14} does not internalize Hh to the endocytic compartment because is defective in endocytosis. We therefore suggest that the massive Hh internalization by Ptc to control the gradient is not a requirement for Hh pathway signal transduction.

In Hh signal transduction, the cellular mechanisms that regulate Smo function remain unclear, although the distribution of Ptc/Smo suggests that Ptc destabilizes Smo levels (Alcedo et al., 2000; Denef et al., 2000; Ingham et al., 2000). It has also been proposed that Ptc-mediated Hh internalization changes the subcellular localization of Ptc preventing Smo downregulation (Denef et al., 2000). Furthermore, in cultured cells, Shh induces the segregation of Ptc and Smo in endosomes, allowing Smo signaling, independently of Ptc (Incardona et al., 2002). It is known, however, that binding of Shh to Ptc is not sufficient to relieve the repression of the Hh pathway (Williams et al., 1999).

We show that as in wild-type cells, in the absence of Hh, Ptc\textsuperscript{14} downregulates both Smo levels and Smo activity, while in the presence of Hh, the normal upregulation of Smo occurs. Consequently, Ptc\textsuperscript{14} levels are high at the AP border because upregulation of Ptc by Hh occurs in the absence of internalization of Hh to the degradative pathway. It might then be expected that the high levels of Ptc\textsuperscript{14} not targeted to the degradative pathway would block Smo activity, However, against all predictions, the presence of Hh is still able to release Smo activity in mutant ptc\textsuperscript{14} cells. Thus, there must be a positive mediator of Smo activity to overcome the repressive effect of Ptc\textsuperscript{14} and allow Hh pathway activation in response to Hh. Alternatively, if Ptc\textsuperscript{14} is located at the plasma membrane, it could control Smo activity without entering the endocytic compartment by regulating the entrance of small molecules, as has been recently proposed (Taipale et al., 2002). In fact, Ptc is similar to a family of bacterial proton-gradient-driven transmembrane molecule transporters known as RND proteins (Tseg et al., 1999). Accordingly, as a membrane transporter, Ptc could indirectly inhibit Smo through translocation of a small molecule that conformationally regulates the active state of Smo (Tseg et al., 1999). The inter-allelic complementation of Ptc suggests that Ptc has the oligomeric structure needed for this type of transporter.

Although one of the normal functions of Ptc is to mediate Hh internalization, the data demonstrate the presence of internalized Hh vesicles in the absence of Ptc protein. It is therefore suggested that another receptor mediates Hh internalization in Hh-receiving cells. This molecule could act as a positive mediator of Hh signaling. Several observations have been published that cannot easily be reconciled with the idea of Ptc acting as the only receptor for Hh. For example, it was found that Hh activates signal transduction in both A and P compartment cells of wing imaginal discs, despite the absence of Ptc in P cells (Ramirez-Wei ber et al., 2000; Amanai and Jiang, 2001). Furthermore, it has been reported that some neuroblasts in Drosophila embryos, the maturation of which is dependent on Hh, do not express or require Ptc (Bhat and Schedl, 1997). This suggests that a receptor other than Ptc mediates Hh signaling. Recently, the glyptic protein Dally-like, which belongs to the heparan sulfate proteoglycan family, was found to be required for Hh signal transduction and probably for the reception of the Hh signal in Drosophila tissue culture cells (Desbordes and Sanson, 2003; Lum et al., 2003).

Dally-like could act as co-receptor for Hh and it would be interesting to know if Dally-like is required for Hh endocytosis. In addition, the large glycoprotein ‘Megalin’ has recently been identified as Shh-binding protein (McCarthy et al., 2002). Megalin is a multi-ligand-binding protein of the low-density lipoprotein (LDL) receptor family whose function is to mediate the endocytosis of ligands (Argraves, 2001) (reviewed by Christensen and Birn, 2002). The finding that megalin-mediated endocytosed N-Shh was not efficiently targeted to lysosomes for degradation suggests that N-Shh may also traffic in complexes with Megalin and thus be recycled and/or transcytosed (Argraves, 2001). In the Wg pathway, specific LDL receptor-related proteins are essential co-receptors for Wnt ligands (Wehrli et al., 2000). Further investigation will determine whether LDL receptor-related proteins could function as co-receptors that internalize Hh in the absence of Ptc. Alternatively, these proteins could be required for endocytosis and further delivery of Hh to Ptc in intracellular vesicles, perhaps facilitating the transcytosis of Hh. A future challenge will be to find other molecules that internalize Hh and to understand how Hh interacts with Smo to activate the Hh pathway.

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