**rap**, a putative transmembrane acyltransferase, is required for Hedgehog signaling

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SUMMARY

Members of the Hedgehog (Hh) family encode secreted molecules that act as potent organizers during vertebrate and invertebrate development. Post-translational modification regulates both the range and efficacy of Hh protein. One such modification is the acylation of the N-terminal cysteine of Hh. In a screen for zygotic lethal mutations associated with maternal effects, we have identified *rasp*, a novel *Drosophila* segment polarity gene. Analysis of the *rasp* mutant phenotype, in both the embryo and wing imaginal disc demonstrates that *rasp* does not disrupt Wnt/Wingless signaling but is specifically required for Hh signaling. The requirement of *rasp* is restricted only to those cells that produce Hh; *hh* transcription, protein levels and distribution are not affected by the loss of *rasp*. Molecular analysis reveals that *rasp* encodes a multipass transmembrane protein that has homology to a family of membrane bound O-acyl transferases. Our results suggest that Rasp-dependent acylation is necessary to generate a fully active Hh protein.

Key words: *rasp*, *hedgehog*, Acyltransferase, Wing imaginal disc, *Drosophila*

INTRODUCTION

Hh proteins make up a conserved family of secreted signaling molecules required during both vertebrate and invertebrate development. These potent signals play a role in specifying cell fate and establishing pattern in many distinct developmental contexts (Hammerschmidt et al., 1997; Chuang and Kornberg, 2000). The most extensively characterized mammalian ortholog is Sonic Hedgehog (Shh), which is required for embryonic segmentation, imaginal disc development and abdominal patterning. The potency of Hh as a global determinant of pattern is illustrated by considering the consequences of Hh misexpression in the *Drosophila* wing imaginal disc (Basler and Struhl, 1994). Many of the basic parameters of the epithelium, such as cell fate, cell number; wing size and morphology are all affected by ectopic Hh expression. Thus, stringent regulation of Hh signaling during development is essential for proper patterning to occur.

One emerging generalization is that Hh patterns a tissue through the establishment of discrete organizing centers (Lawrence and Struhl, 1996). Hh can act both as a short-range inducer and as a long-range morphogen in patterning. For example, in the *Drosophila* wing imaginal disc epithelium Hh is produced in presumptive posterior cells under the control of *en grailed* (*en*) (Zecca et al., 1995), a transcription factor, that functions as a ‘selector’ gene for posterior identity. En also prevents posterior cells from responding to the Hh signal (Sanicola et al., 1995; Tabata et al., 1995; Zecca et al., 1995). Consequently, only anterior cells respond to Hh by activating target genes in a narrow stripe adjacent to the anteroposterior (AP) boundary. One such target is *decapentaplegic* (*dpp*), which encodes a morphogen that can signal over a long range to specify cell fates in both the anterior and posterior of the wing (Lecuit et al., 1996; Nellen et al., 1996). However, in other contexts, Hh itself can act as a long-range morphogen, as shown in the adult abdomen (Struhl et al., 1997a; Struhl et al., 1997b). Specific regulation of both the range of Hh movement and Hh activity at organizing centers will therefore determine many aspects of a tissue’s overall pattern.

The post-translational modification of Hh appears to be crucial in regulating both the range of Hh signaling and its activity. Biochemical studies suggest that two distinct activities reside within the full-length precursor form of Hh. The N-terminal fragment is required for signaling, and is liberated from the C terminus by an intramolecular cleavage event (Lee et al., 1994; Porter et al., 1995; Porter et al., 1996a). The C terminus possesses a cholesterol transferase activity, and is sufficient to catalyze the covalent addition of a cholesterol moiety to the N-terminal fragment of Hh (Porter et al., 1996b). Cholesterol modification of Hh is not, however, a pre-condition for generating an active Hh species, as expression of mutant
forms of Hh that cannot be modified retain the ability to signal (Porter et al., 1996a; Burke et al., 1999). Moreover, the unmodified N-terminal fragment of Drosophila Hh is capable of signaling over an even longer range than its cholesterol-modified counterpart (Porter et al., 1996a; Burke et al., 1999). By contrast, long-range signaling of Shh appears to require cholesterol modification (Lewis et al., 2001). Thus, addition of cholesterol appears to be a key factor in regulating the range of Hh signaling.

There is also evidence that Hh proteins bear at least one additional lipid modification. Mass analysis of full-length human Shh expressed in insect cells showed that the total mass of purified Shh could not be accounted for solely by the addition of cholesterol (Pepinsky et al., 1998). This observation suggested that Shh might be fatty acylated. Palmitoylated proteins contain a 16-carbon saturated fatty acyl group that is almost always attached to a cysteine residue by a thioester bond. Pepinsky et al. have observed that the mass of a palmitoyl moiety is sufficient to account for the difference between the calculated and measured mass of Shh (Pepinsky et al., 1998). Direct evidence for palmitic acid modification has come from monitoring the level of radiolabeled palmitic acid incorporation into Shh. While Shh was readily labeled, a variant in which Cys-24 was mutated to Ser-24 showed no palmitic acid incorporation (Pepinsky et al., 1998). Hence, Cys-24, the N-terminal amino acid of Shh, is specifically required for palmitoylation.

Recent experiments have now begun to address the function of fatty-acylated Hh proteins (Kohtz et al., 2001; Lee et al., 2001). These studies sought to compare the activity of wild-type Hh with mutant forms of Hh that lacked the N-terminal Cys, in vivo. In one assay, the ability of Shh to induce rat telencephalic neurons was dependent upon the presence of an N-terminal Cys (Kohtz et al., 2001). Similarly, the activity of Hh in patterning the wing of Drosophila was shown to depend on the presence of an N-terminal Cys (Lee et al., 2001). Together, these experiments suggest that fatty-acylation is critical for regulating Hh activity in vivo.

We describe the molecular cloning and characterization of rasp, a novel Drosophila segment polarity gene. We demonstrate that the function of rasp is specific for Hh signaling. Furthermore, we show that the requirement for rasp is spatially restricted and limited only to hh-expressing cells. Loss of rasp activity does not disrupt hh transcription, nor does it affect Hh protein levels or distribution. Molecular analysis reveals that rasp encodes a putative multipass transmembrane protein with homology to a conserved family of membrane bound O-acyl transferases (MBOAT) (Hofmann, 2000). We propose that Rasp, like the other members of the MBOAT family, acts as an acyltransferase and is required for fatty-acylate Hh, a modification required for Hh signaling.

**MATERIALS AND METHODS**

**Drosophila stocks**

*ovo*Δ1, FRT2A (Chou and Perrimon, 1996). y w; rasp FRT2A, FRT82B, w; rasp FRT2A, FRT82B; and y w; trh FRT2A, FRT82B (this study). y w hsflip, ßMyc FRT80 (kindly provided by S. Blair), y w hsflip, M(3)G5 hsGFP FRT74 (kindly provided by G. Struhl), en Gal4, UAS-GFP, ptc Gal4, UAS-GFP, UAS-hh FRT1A (Burke et al., 1999) (kindly provided by K. Basler). Rasp is a transgene that generates a full-length Hh with an HA tag. UAS-hh and UAS-hh N (kindly provided by P. Beachy). hh183 (lacZ) (Lee et al., 1992), ptc- lacZ and dpp109 (lacZ) (kindly provided by S. Cohen). ci-DipE (Eaton and Kornberg, 1990). hhGal4 (Tannimoto et al., 2000) (kindly provided by Tetsuya Tabata). FRT135 mCD8 GFP (Lee and Luo, 1999).

In a genetic screen for maternal/zygotic genes (N. P. C. Arnold and A. Lanjuin, unpublished) we have identified two rasps alleles, rasp and raspΔ1. These mutations were induced on a y w; FRT2A FRT82B chromosome (Chou and Perrimon, 1996). An additional allele, l(3)63Bg, was subsequently identified in the Bloomington stock center collection by complementation testing. The phenotypes of each or these three mutations were tested over a small deficiency in the region and found to be similar to the homozygous phenotype of each allele alone. Furthermore, the molecular lesion associated with raspΔ1 is consistent with this allele being a protein null, as it introduces a premature stop codon into the predicted protein (see Results). The rasp and raspΔ1 phenotypes we observe are similar to those previously described for l(3)63Bg (Woldwll and Bronner, 1991). In the present analysis, we used raspΔ1 in the generation of marked clones and raspΔ1 to generate discs homozygous for rasp.

**Clonal analysis**

Germline clones (GLC) were induced using the FLP-FRT dominant female sterile method as previously described (Chou and Perrimon, 1996). Recombinant somatic clones were generated by collecting eggs for 3 days and then administering a 37°C heat shock for 1–2 hours (depending on the intended rate of clone induction). To obtain the cohort of larvae that received a heat shock at the beginning of second instar, white prepupae (wpp) were picked from the bottle 72 hours after clone induction. The wpp were transferred to a moist plastic petri dish and heat shocked at 37°C to induce marker gene expression (1 hour for Myc, 2 hours for GFP). Wpp were immediately dissected and prepared for staining.

**Histology and immunohistochemistry**

For most antibody stains, discs were dissected in Drosophila ringers and fixed in ‘Browers’ buffer + 2% EM grade formaldehyde overnight at 4°C (for details, see Blair, 2000). For anti-Hh staining we found that the protocol described by Burke et al. (Burke et al., 1999) produced the most reliable results. For in situ hybridization to RNA probes, discs were fixed in PBS + 8% formaldehyde and processed according to Hauptmann (Hauptmann, 2001).

Primary antisera used were: Anti-Hh (gift from Ingham), 1:1000; Anti-Hh (gift from Kornberg), 1:10,000; anti-Ptc (gift from I. Guerrero) 1:1000; anti-Sc (gift from S. Carroll) 1:1000; anti-Dll (gift from S. Cohen), 1:1000; anti-Wg (gift from S. Cohen), 1:10; anti-Myc (gift from S. Blair), 1:4; anti-En/Inv (gift from E. Spana), 1:4; anti-En/Inv (gift from N. Patel), 1:10; anti-ß-gal (Cappell) 1:200; anti-HA (Roche), 1:1000; anti-Crums (gift from E. Knust), 1:50; and anti-Digalkaline phosphatase (Roche), 1:200. Fluorescent secondary antibodies (low cross reactivity from Jackson ImmunoResearch Labs) were used at 1:200 and incubated at 4°C overnight. In some cases, discs were stained using a nickel-intensified Vector ABC-DAB protocol as described by Blair (Blair, 2000). Discs were mounted in Vectashield mounting media and inspected using a Leica TCS-NT confocal microscope. Single images were merged using Adobe Photoshop.

**Molecular biology**

The ORF corresponding to CG11495 was amplified from genomic DNA by PCR using specific oligonucleotide primers. PCR products were cloned using the Promega pGemEasy kit. Genomic DNA was prepared according to a modified form of the single fly PCR protocol as described by Burke et al. (Burke et al., 1999). To identify mutations, DeepVent DNA polymerase (New England Biolabs) was used to...
amplify CG11495 fragments from both y w; rasp^{7F21} FRT^{2A} FRT^{82B} and y w; FRT^{2A} FRT^{82B} genomic DNA. rasp^{7F21} homozygous embryos were selected from a y w; rasp^{7F21} FRT^{2A} FRT^{82B} /TM6, GFP stock under a fluorescent dissecting microscope. For each genotype, the PCR products from each of three independent, but identical, reactions were sequenced and compared. DNA star software suite was used for compiling sequences, generating alignments and analyzing predicted protein structure. RNA probes were generated according to Hauptmann (Hauptmann, 2001).

RESULTS AND DISCUSSION

rasp mutant embryos display a segment polarity phenotype

In a large genetic screen for EMS-induced, zygotic lethal mutations with maternal effects (N. P., C. Arnold and A. Lanjuin, unpublished), we identified two allelic mutations on the third chromosome: 7F21 and 9B15. Normally, the ventral cuticle of a wild-type embryo displays a segmentally repeating pattern of denticle belts and naked cuticle. By stage 11, three invaginations of the foregut form in the dorsal epithelium and are easily visualized using the epithelial marker Crumbs. Loss of Wg signaling leads to the formation of only a single invagination, while SNS development is not disrupted in hh mutants (González-Gaitán and Jäckle, 1995). rasp mutant embryos stained with anti-Crumbs showed no defects in SNS formation (data not shown). Thus, Rasp is not required for the Wg-dependent patterning of the SNS.

We next extended our analysis to the wing imaginal disc, where Wg and Hh signaling pathways also have distinct patterning requirements. In the wing, Wg acts over a long range to pattern the dorsoventral (DV) axis of the wing (Zecca et al., 1996; Neumann and Cohen, 1997), while Hh patterns the AP axis (Basler and Struhl, 1994; Strigini and Cohen, 1997), where Wg and Hh signaling pathways also have distinct patterning requirements. In the wing, Wg acts over a long range to pattern the dorsoventral (DV) axis of the wing (Zecca et al., 1996; Neumann and Cohen, 1997), while Hh patterns the AP axis (Basler and Struhl, 1994; Strigini and Cohen, 1997). By late third instar, wg is expressed in a narrow stripe three to five cells wide along the presumptive wing margin (Baker, 1988a). Wg is necessary and sufficient to activate both proneural gene expression (Blair, 1992b; Phillips and Whittle, 1993) and Distalless (Dll) expression (Zecca et al., 1996; Neumann and Cohen, 1996) within the wing pouch. We used a GFP marked Minute chromosome to generate large rasp– clones and examined protein levels of both the proneural gene scute (sc) and Dll (Fig. 2A–F). None of the clones examined resulted in a discernable disruption of either the level or pattern of Sc (Fig. 2A–C) and Dll (Fig. 2D–F) proteins. This was true even for early clones that straddled the DV boundary. Taken together, our data show that rasp is not required for Wg signaling.
rasp is required for activation of Hh target genes

We next examined the requirement of rasp for Hh target gene activation. In the wing disc patched (ptc) (Phillips et al., 1990) and dpp (Masucci et al., 1990) are expressed in a narrow anterior stripe along the AP boundary (Fig. 3B,C). Of these, ptc expression requires higher levels of Hh signaling (Capdevila et al., 1994; Strigini and Cohen, 1997). In wing discs homozygous for rasp, the level of ptc expression was reduced, as shown by both the ptc-lacZ (Fig. 4B) and ptc Gal4, UAS-GFP reporters (data not shown). A striking feature of the rasp wing disc phenotype is their markedly reduced size (compare Fig. 4A with 4B). This suggests that dpp expression might also be reduced in this mutant background, as reception of dpp is normally required for cell proliferation within the wing disc (Burke and Basler, 1996). In situ hybridization of RNA probes to wing discs homozygous for rasp revealed a reduction in dpp expression levels (compare Fig. 4C with 4D).

Thus, rasp activity is necessary for the expression of two direct transcriptional targets of Hh.

Hh signaling is also required to maintain distinct populations of anterior and posterior cells in the wing imaginal disc. Failure of anterior cells to receive Hh results in a loss of target gene expression and a distortion of the AP lineage restriction (Blair and Ralston, 1997; Rodriguez and Basler, 1997); distortion of the AP lineage boundary is thus another measure of Hh signaling loss. We observe that in wing discs stained with anti-En/Inv (brown) the domain of elevated ptc expression can be detected in anterior cells by late third instar (purple). The expression of two additional Hh target genes can be detected in anterior cells; (B) ptc-lacZ, is shown in blue and (C) dpp-lacZ, is shown in yellow. Brackets highlight the domains of anterior Hh target gene expression.

![Fig. 2. Loss of rasp activity does not affect Wg signaling.](image)

(A-F) Confocal micrographs of late third instar wing imaginal discs containing rasp7F21 (rasp) clones. In this and all subsequent micrographs, anterior is upwards and dorsal is on the left. Large rasp− clones that cross the DV boundary were generated in a Minute heterozygous (M+) background and are marked by the absence of green fluorescent protein (GFP). (C,F) Broken line marks the approximate position of the DV boundary. (A) The absence of GFP in green marks the tissue homozygous for rasp. (B) The double row (DR) of anti-Sc staining shown in red marks the anterior proneural region along the presumptive wing margin. Activation of proneural gene expression along the anterior margin depends on Wg signaling. (C) Overlay of A and B. Note that anti-Sc levels and pattern within the clone are not affected by loss of rasp. (D) Absence of GFP in green marks tissue homozygous for rasp. (E) Dll is normally expressed throughout the majority of the presumptive wing pouch and is delimited by a broken line. Activation of Dll expression in the wing pouch depends on Wg signaling. (F) Overlay of D,E. Note that anti-Dll levels within the clone are not affected by loss of rasp.

![Fig. 3. Hh-dependent gene expression along the AP boundary.](image)

(A) Anterior cells are marked by the expression of ci, shown in red. Posterior cells are marked by anti-En/Inv staining shown in blue. A broken line marks the approximate position of the AP boundary. en is required in posterior cells for hh expression; hhGal4 UAS-GFP is shown in green. Anterior cells adjacent to the AP boundary respond to Hh by activating target gene expression. A narrow, Hh-dependent stripe of anti-En/Inv staining can be detected in anterior cells by late third instar (purple). The expression of two additional Hh target genes can be detected in anterior cells; (B) ptc-lacZ, is shown in blue and (C) dpp-lacZ, is shown in yellow. Brackets highlight the domains of anterior Hh target gene expression.

![Fig. 4. rasp is required for Hh target gene activation.](image)

(A-D) Comparison of Hh target gene expression in wing discs heterozygous and homozygous for rasp. (A) ptc-lacZ; rasp9B15/TM6 discs stained with anti-β-gal (blue) and anti-En (brown). The domain of elevated ptc expression is detectable in a narrow stripe of anterior cells along the AP boundary (arrow). (B) ptc-lacZ; rasp9B15/rasp9B15 discs stained with anti-β-gal (blue) and anti-En (brown). Levels of ptc-lacZ are reduced in this mutant background (compare A with B). The anti-β-gal stain was amplified to detect lower levels of ptc-lacZ reporter signal (see Materials and Methods). We failed to detect ptc-lacZ expression using fluorescent secondary antibodies. Note the reduced size of the homozygous wing disc. (C) ptc-lacZ; rasp9B15/TM6 discs hybridized to dpp RNA probes (blue). Like ptc-lacZ, dpp is also expressed in a stripe of anterior cells along the AP boundary (arrow). (D) ptc-lacZ; rasp9B15/rasp9B15 discs hybridized to dpp RNA probes (blue). Levels of dpp transcripts are reduced in this mutant background (compare C with D).
discs homozygous for rasp, the AP boundary, as defined by en expression, occasionally appears to be distorted (data not shown). Taken together, we conclude that rasp is required for Hh signaling.

**rasp is required in cells that send the Hh signal**

Reductions in the level of Hh target gene expression can be observed in mutations that disrupt either the process of sending or receiving Hh. Using clonal analysis, we tested the requirement of rasp for Hh target gene activation in both sending and receiving cells. Large rasp− clones that were restricted to the anterior compartment and defined the AP boundary, had no detectable effect on Ptc protein levels (Fig. 5A-C). Anterior En protein levels were also examined in this assay; by late third instar, a Hh-dependent stripe of En expression is present along the AP boundary (Blair, 1992a; Blair and Ralston, 1997). The level of anterior En protein was similarly unaffected by large anterior rasp− clones (Fig. 5G-I). Anterior clones distant from the AP boundary also had no effect on either Ptc or En levels. By contrast, large rasp− clones restricted to the posterior compartment resulted in a non-autonomous reduction in the levels of both Ptc and anterior En protein levels (Fig. 5D-F,J-L). These effects were most pronounced in large posterior clones abutting the AP boundary. Posterior rasp− clones that were small or distant from the AP boundary did not result in detectable reductions of either Ptc or En protein. Interestingly, we observed that the presence of wild-type posterior tissue adjacent to the AP boundary could often rescue anterior Hh target gene expression, even when the remaining posterior cells were rasp negative (data not shown). Hence, rasp is required only in posterior cells, and is both necessary and sufficient for anterior Hh target gene expression along the AP boundary.

Previous observations have suggested that Hh produced in the posterior compartment can travel over a long range to activate target gene expression in cells of the anterior compartment (Chen and Struhl, 1996). The non-autonomous effects we observe in large posterior rasp mutant clones are therefore consistent with a role for rasp in Hh signaling. One explanation for our observations is that rasp is required for hh transcription in posterior cells. To test this possibility, we examined hh transcription in posterior rasp clones using a hh-lacZ reporter. No effect on hh-lacZ levels was observed (Fig. 6A-C). Thus, hh transcription does not require rasp activity.

Non-autonomous defects in Hh target gene expression have also been observed in mutations that are required for sending the Hh signal. This is the case for posterior dispatched (disp) mutant clones where a failure to secrete Hh from sending cells correlates with an autonomous increase in Hh protein levels (Burke et al., 1999). We examined Hh protein in posterior rasp− clones and found that, in general, Hh protein levels were neither elevated nor reduced compared with wild-type cells (Fig. 6D-F). However, we note that in a few cases there did appear to be a detectable increase in Hh protein levels within rasp mutant cells (data not shown). We also examined the apical-basal axis of wing disc epithelium and found no discernable effect on the subcellular distribution of Hh (data not shown). Therefore, the primary defect in rasp mutants does not appear to be due to a failure of Hh secretion, a reduction in Hh protein stability or mislocalization of Hh protein. We conclude that in the absence of rasp, Hh protein is likely to be properly secreted from posterior cells but that it cannot activate the Hh pathway in receiving cells.
rasp is required to produce a fully active Hh protein

Activation of anterior Hh target gene expression requires that Hh protein produced by posterior cells is both active and that it is transported to anterior receiving cells. The processes of Hh signaling and transport have been experimentally separated. For example, overexpression of Hh in anterior cells results in the activation of Hh target gene expression even in adjacent cells (Strigini and Cohen, 1997; Burke et al., 1999), while activation of a membrane-tethered form of Hh results in target gene activation only in adjacent cells (Strigini and Cohen, 1997; Burke et al., 1999). Furthermore, mutations in tout-velu disrupt long range Hh transport, but not the responsiveness of anterior cells to Hh (Bellaiche et al., 1998).

If the function of rasp is required for Hh activity and not for transport, then overexpression of Hh in a rasp mutant background should fail to activate Hh target gene expression. Alternatively, if rasp is required for the transport of Hh, then overexpression of Hh in a rasp mutant background should lead to target gene activation only in adjacent cells. To address this issue, we expressed a full-length, tagged form of Hh (Burke et al., 1999) in posterior cells that were either heterozygous or homozygous for rasp. Hh-N is not detectable (compare C with D). Note that the anterior expression of en is also not detectable in these discs. (E) Higher magnification of disc shown in D. (F) UAS-Hh N/+; en Gal4, UAS-GFP/+; rasp

rasp encodes a putative multipass transmembrane protein with homology to a conserved family of acyltransferases

To molecularly characterize rasp, we first determined its location on the third chromosome. Deficiency mapping revealed that rasp was localized to a region between 63A1 and 63D1, as rasp

To map the location of rasp more finely, we performed complementation tests with available mutations in the region. We identified one mutation, l(3)63Bg, that failed to complement rasp and had a similar mutant phenotype (Wohllwill and Bronner, 1991). We next measured the recombination rates between rasp and three lethal P-elements in the region, l(3)06803, l(3)j5C2 and l(3)01029. No recombinants between rasp and l(3)j5C2 were recovered in our experiment, suggesting a map position for rasp close to 63B13.
Examination of the information from the Berkeley Drosophila Genome Project (BDGP) revealed the presence of a number of putative open reading frames (ORFs) in the region of 63B13. Furthermore, through personal communication with K. Basler, we became aware that his group was also characterizing a segment polarity gene in the region that failed to complement l(3)63Bg and that corresponded to ORF CG11495, located at 63B11. To determine whether rasp was a mutation underlying CG11495, we compared the genomic sequence of the parental, unmutagenized chromosome used in mutation underlying CG11495, we compared the genomic sequence of the parental, unmutagenized chromosome used in our screen with the genomic sequence of rasp\textsuperscript{7F21}. We found that rasp\textsuperscript{7F21} was associated with a C to T base substitution that would introduce a premature stop codon at amino acid 52 of the predicted protein encoded by CG11495 (Fig. 8, asterisk). DNA amplified from CG11495 was cloned and used to generate RNA probes. As predicted, based on the domain of Hh responsiveness, rasp transcripts were detected ubiquitously at low levels throughout the wing imaginal disc (data not shown).

Sequence analysis of the predicted protein revealed that Rasp is quite hydrophobic and contains at least eleven membrane-spanning regions (Fig. 8). We also identified an invariant histidine residue that may mark the position of the active site. Members of a conserved family of membrane bound MBOAT proteins have two characteristics in common. First, these proteins typically contain between eight and ten membrane-spanning regions. Second, they share a region of sequence similarity in common that includes an invariant histidine residue within a long hydrophobic region (Hofmann, 2000). Based on these criteria, Rasp appears to be a bona fide MBOAT protein.

In this study, we have described the requirement of rasp in patterning the Drosophila embryo and wing imaginal disc. Our results indicate that rasp is required for Hh signaling and that post-translational lipid modification is crucial for long and short range, Hh-dependent patterning. These conclusions are based on the following observations:

1. We have examined the requirement of rasp for both Wg and Hh signal transduction. Our experiments show that loss of rasp does not alter Wg target gene expression; however, the expression of direct transcriptional targets of Hh signaling are reduced in the absence of rasp.

2. Using clonal analysis, we have tested the requirement of rasp in Hh-sending and -receiving cells. We observe that loss of rasp in posterior, Hh-sending cells, has a non-autonomous effect on Hh target gene expression, while no requirement for rasp in receiving cells was detected.

3. hh transcription, protein levels and distribution do not depend on rasp function.

4. rasp is required for the production of an active Hh protein.

5. rasp encodes a protein with homology to acyltransferases.

What, then, is the role of Rasp in modifying the Hh protein? One possibility is that Rasp is required directly for the cholesterol modification of Hh, and that rasp mutant cells produce Hh, but that it lacks a cholesterol moiety. Elegant studies in Drosophila have examined the role of cholesterol modification for Hh activity. These experiments demonstrate that Hh proteins, which are not cholesterol modified, can still activate Hh target genes, even in the absence of endogenous Hh (Porter et al., 1995; Burke et al., 1999). This, however, is not consistent with what we observe, as lack of rasp activity leads to a reduction of target gene expression. Therefore, our data do not appear to be consistent with a role for rasp in cholesterol modification.

Members of the MBOAT superfamily that have been well-characterized biochemically encode enzymes that transfer fatty acids onto hydroxyl groups of membrane-tethered targets. Palmitoylation, the attachment of saturated 16-carbon fatty acyl chain to a protein by a thioester bond, is the only other lipid modification of Hh that has been described (Pepinsky et al., 1998). Thus, if Rasp does palmitoylate Hh, it is possible that Rasp is required for additional lipid modifications that are required for Hh activity.

Among acylated proteins, there are a growing number that require additional lipid modifications that are required for Hh activity, but have yet to be characterized.

Among acylated proteins, there are a growing number that regulate developmental signaling events. Our study has highlighted the importance of lipid modification for Hh signal transduction. Such lipid modifications appear to be important.

**Fig. 8.** rasp encodes a putative multipass transmembrane protein with homology to acyltransferases. (A) Sequence analysis of rasp. Hydrophobicity plot shows that Rasp encodes a predicted protein that is highly hydrophobic. At least eleven transmembrane regions can be detected. (B) Linear sequence of amino acids predicted by the BDGP rasp sequence. Hydrophobic regions are indicated in bold. Sequencing genomic DNA from rasp\textsuperscript{7F21} homozygous embryos led to the identification of a single base pair change that converts a tryptophan to a premature stop codon at amino acid 52 (asterisk). An invariant histidine residue (underline) conserved among MBOAT family members is a likely candidate for the active site of Rasp.
for protein-membrane interactions and for targeting proteins to membrane microdomains (Casey, 1995). Palmitoylation, in particular, has been the subject of increasing interest (Mumby, 1997; Resh, 1999). This may be due to the fact that, unlike other lipid modifications, palmitoylation can be reversible and thus, constitutes a regulatable step in signal transduction. Finally, like rasg, porcupine (porc) also encodes a protein with homology to members of the MBOAT superfamily (Hofmann, 2000); porc is required for sending the Wg/Wnt signal (Kadowaki et al., 1996). Thus, some acylating enzymes appear to be highly specific, and may prove to be of general importance in regulating the function of secreted proteins during development.

Note added in proof
As we were preparing this manuscript for publication, the molecular and phenotypic analysis of sightless (sit) (Lee and Triesman, 2001) and skinny hedgehog (shk) (Chamoun et al., 2001) were reported. sit, ski and rasp correspond to the same gene and our findings are in agreement.

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