**Drosophila** Wnt-1 Undergoes a Hydrophobic Modification and Is Targeted to Lipid Rafts, a Process That Requires Porcupine*

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Wnt signaling pathways regulate many developmental responses; however, little is known about how Wnt ligands function on a biochemical level. Recent studies have shown that Wnt-3a is palmitoylated before secretion. Here we report that *Drosophila* Wnt-1 (Wingless) also undergoes a lipid modification. Lipidation occurs in the endoplasmic reticulum and is dependent on Porcupine, a putative O-acyltransferase. After modification, DWnt-1 partitions as a membrane-anchored protein and is sorted into lipid raft detergent-insoluble microdomains. Lipidation, raft targeting, and secretion can be blocked by the addition of 2-bromopalmitate, a competitive inhibitor of O-acyltransferase activity. Based on these results we propose a model whereby lipidation targets Wnt-1 to secretory vesicles that deliver the ligand to specialized microdomains at the cell surface where it can be packaged for secretion.

Wnt ligands play key roles in many developmental pathways (1–3). To understand how Wnts regulate so many developmental activities, it is necessary to define the biochemical steps that constitute the actual signal transduction events. With this goal in mind, we are studying the synthesis and secretion of active Wnt-1 ligand in *Drosophila melanogaster*. The Wnt genes encode a large family of secreted proteins; these ligands share a signature WNT motif (C-K-C-H-G-(LIVMT)-S-G-(D/E)), 22 conserved cysteines, many highly charged amino acid residues, and several potential glycosylation sites. Based on their amino acid sequences, Wnt proteins should be soluble, secreted glycoproteins; yet, Wnts do not exhibit the properties expected of soluble hydrophilic proteins. For example, we have examined *Drosophila* Wnt-1 expression in transgenic S2 cells and found that only about 20% of the secreted protein is present in soluble conditioned medium; the majority of the extracellular Wnt-1 is associated with the cell surface and extracellular matrix (4). Recent work by Nusse and co-workers (5) has shown that murine Wnt-3a is palmitoylated at a conserved cysteine residue (Cys-77). Their discovery suggests for the first time that lipid modifications may account for the unusual behavior of Wnt ligands. How palmitoylation affects Wnt-3a signaling and whether or not other Wnts are modified in a similar manner are questions now under investigation. Here, we provide evidence that *Drosophila* Wnt-1 is also lipid-modified. In addition, we investigate the functional significance of the lipid modification and the role of porcupine in Wnt-1 maturation and secretion.

Many types of proteins (including cytosolic, transmembrane, and secreted proteins) are known to undergo S-palmitoylation, the reversible addition of palmitate to a cysteine via a thioester bond (6). This posttranslational acylation is readily reversible and can regulate both protein localization and function. The addition of the palmitoyl moiety increases protein hydrophobicity and promotes membrane association (6). Palmitoylation also affects intracellular trafficking in that palmitoylated proteins are frequently targeted to specific intracellular organelles as well as to detergent-resistant microdomains (DRMs) located at the plasma membrane (7, 8). These DRMs, commonly referred to as lipid rafts, are rich in cholesterol and glycosphingolipids and so exist in a separate liquid-ordered phase within the plasma membrane (9). Raft DRMs often form signal transduction centers. The cellular machinery needed for signal transduction becomes organized as some proteins are targeted to rafts, whereas others are excluded. For example, cell surface receptors are sometimes localized to same membrane microdomains where their downstream intracellular partners are also concentrated (10).

Secreted proteins can also be palmitoylated. The mature form of Hedgehog, Hh-Np, contains two unusual lipid modifications (a C-terminal linked cholesterol moiety and an N-terminal palmitoyl adduct). These lipid modifications increase Hh-Np hydrophobicity, influence apical sorting of the ligand, and likely play a role in the partitioning of Hh-Np into raft DRMs (11–14). Skinny Hedgehog, a putative O-acyltransferase, is required for the palmitoylation of *Drosophila* Hh-Np (15). Clonal analysis studies (12) suggest that palmitoylation is essential for Hh signaling in the wing disc. In the absence of Skinny Hedgehog activity, Hh-Np is not palmitoylated, and Skinny Hedgehog signaling activity is greatly reduced.

Skinny Hedgehog belongs to a diverse family of membrane-bound O-acyltransferases (16). Many of these enzymes catalyze the transfer of fatty acids to hydroxyl groups on hydrophobic targets. The target substrates vary widely and include lipids (cholesterol acyltransferase), alginate (AlgI), and waxes (wax synthase). Skinny Hedgehog is somewhat unusual in that it is required for the synthesis of a thioester-linked fatty acid. The chemistry of S-palmitoylation and the palmitoyl acyltransferase enzymes have not been well studied. Some palmitoyl acyltransferases reside in the cytosol; others, like Skinny Hedgehog, are thought to function within the ER (6, 15). Curiously, the vertebrate Sonic Hedgehog protein, Shh-Np, con-

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§ The abbreviations used are: DRM, detergent-resistant microdomain; ER, endoplasmic reticulum; PCNA, proliferating cell nuclear antigen; PBS, phosphate-buffered saline; CHAPS, 3-[3-cholamidopropyldimethylammonio]-1-propanesulfonic acid; DFz2, DFrizzled2; Syy1A, syntaxin 1A; porc, porcupine; Hh, hedgehog; Drac-1, Drosophila Rac-1.
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Tepains an amide-linked palmitoyl group at the N-terminal Cys. Pepinsky et al. (11) have suggested that Shh-Np acylation occurs via a two-step mechanism, which is the initial synthesis of a thioester intermediate linking the palmitoyl group to the side chain of the terminal cysteine followed by an intramolecular rearrangement that transfers the palmitoyl group to the N-terminal nitrogen (11). The presence of the amide bond may be functionally significant because an amide bond is far more stable than a thioester linkage.

It is intriguing that porcupine also encodes a putative multi-tap transmembrane protein belonging to the membrane-bound O-acyltransferase superfamily (16, 17), porcupine is required for Wnt-1 activity, and porcupine homologs have been identified in Xenopus, mouse, human, and Caenorhabditis elegans (18–20). Genetic and immunocytotoxic studies suggest that porcupine is required for the secretion of active Wnt-1 ligand, porcupine functions upstream and is required by Wnt-1 expressing cells. Wnt-1 is synthesized in porcupine± mutants; however, it accumulates within the expressing cells. All extra-cellular Wnt-1 staining is absent, suggesting that Wnt-1 secretion is blocked in the porcupine± mutant animals (16, 21). The Wnt-1 secretion defect can be bypassed by driving overexpression of Wnt-1. In this case, ectopic Wnt-1 secretion leads to ectopic signaling and death. Thus porcupine has an essential role in directing proper Wnt-1 secretion. Studies with transgenic S2 cells (18, 22) have shown that Porcupine and the N-terminal portion of Wnt-1 can be co-immunoprecipitated in the same complex, suggesting that Porcupine may interact directly with Wnt-1. Several questions should now be addressed. Does Porcupine mediate a posttranslational modification of Wnt-1? If so, what is the modification, and why is this modification necessary for normal Wnt-1 secretion?

Here, we report a series of experiments testing the hypothesis that porcupine is required for lipidation of Wnt-1. We present evidence that Wnt-1 is lipid-modified and that this modification converts Wnt-1 into a membrane-anchored protein that is partitioned into specialized lipid raft microdomains before secretion. Moreover, we show that Porcupine activity is required for both lipidation and the subsequent targeting of DWnt-1 to specialized raft DRMs at the cell surface.

Materials and Methods

Cell Culture, Protein Induction, and Western Blotting—Drosophila S2 cell lines were cultured as described (4). S2-DWnt-1 cells carry a winglessag cDNA transgene under the control of the inducible Hsp70 promoter; S2-DFz2 cells (provided by Roel Nusse) have a Dfrizzled/Dfz2 cDNA transgene under control of the inducible metallothionein promoter. DWnt-1 expression was induced by heat shock S2-DWnt-1 cells for 1 h at 37 °C followed by a 2-h recovery period at 25 °C. DFz2 expression was induced by treating S2-DFz2 cells with 70 μM CuSO4 for 2 h.

10% SDS-PAGE and Western analysis was performed as described (4). Antibody dilutions used were: mouse α-DWnt-1 (ATCC), 1:1000; mouse α-PCNA (Novacastra Laboratories Ltd., Novus Biologicals), 1:500; rabbit α-Dfz2, 1:1000; mouse α-aspartic acid A1, 1:1000 (Developmental Studies Hybridoma Bank), mouse α-Rac1 1:1500 (BD Biosciences), and mouse α-Hsp70 1:10,000 (Affinity Bioreagents Inc).

Butyl-Sepharose Chromatography—Cells were washed in PBS, resuspended in 2 × 106 cells/ml cold binding buffer (100 mM NaCl, 10 mM Tris-Cl, pH 8.0, 0.025% CHAPS), with 1× protease inhibitors mixture, and centrifuged for 20 min at 80,000 g at 4 °C, which was followed by gentle shaking at 4 °C for 1 h. Supernatant was collected at 37 °C for 8 min and then centrifuged at 10,000 × g at room temperature for 10 min. The aqueous phase and the detergent phase were separated, subjected to 10% SDS-PAGE, and analyzed by Western blotting.

Preparation of Whole Animal Extracts—hs-wg;porc± larvae were generated by crossing porc±;FM7c females to hs-ug:TM6Tb males. Late third instar porc±;hs-ug animals were identified by scoring for non-Tubby, y± male larvae. Two genotypes were used for hs-ug control animals; they are hs-ug;FM7c (obtained from the cross described above) and hs-ug:TM6Tb. Individual larvae were heat-shocked for 1 h at 37 °C and allowed to recover at 25 °C for 2 h. After heat shock and recovery, each animal was lysed individually. For butyl-Sepharose chromatography, each larva was lysed in 100 μl of binding buffer (100 mM NaCl, 10 mM Tris-Cl, pH 8.0, with 1× protease inhibitor mixture) by first homogenizing the animal with a pestle and then passing the extract 20 times through a 0.22-μm syringe, which was followed by centrifugation at 5000 × g for 5 min to remove debris. Crude extracts were subjected to butyl-Sepharose chromatography.

For membrane extraction, crude protein extracts were obtained by homogenizing each larva in cold PBS and 0.05% CHAPS (2 × 105 cells/ml) containing 1× protease inhibitors mixture as described above. After centrifuging at 5000 × g for 5 min to remove debris, the membrane fraction was isolated and subjected to sodium carbonate extraction as described above.

For Triton X-114 phase separation, each larva was lysed in 100 μl of ice-cold Triton lysis buffer by homogenization with a pestle, and then the lysate was passed through a 0.22-μm needle 20 times. Protein extracts were subjected to Triton X-114 phase separation as described above.

Hydroxylamine Treatment of S2-DWnt-1 Whole Cell Extract—5 × 106 cells were collected and lysed in 500 μl of PBS with 1× protease inhibitors mixture. The lysates were mixed with 500 μl of 2 mM Tris/HCl, pH 7.5, or 500 μl of 2 mM hydroxylamine/HCl, pH 7.5, and incubated at 37 °C for 4 h. The samples were then subjected to butyl-Sepharose hydrophobic chromatography and Western blot analyses as described above.

Isolation of Lipid Raft Detergent-resistant Microdomains—Raft DRMs were prepared and isolated as described (24, 25). Briefly, cells were washed in PBS and then suspended in cold TNET buffer (100 mM NaCl, 150 mM Tris, 10 mM EDTA, 1% Triton X-100) with 1× protease inhibitor mixture. Cells were lysed by passing the suspension through a 27-gauge needle 20 times; lysates were centrifuged at 5000 × g for 5 min to remove debris, and the supernatant was diluted to 1:2 (v/v) with OptiPrep™, a 60% (v/v) solution of iodixanol in water (Accurate Chemicals and Scientific Corp.) to reach a final concentration of 40% OptiPrep. Step gradients were formed in Beckman SW80 tubes and the cell lysates were mixed with 10 μl of 40% OptiPrep and 10 μl of 60% OptiPrep. Individual gradients were centrifuged at 40,000 rpm for 4 h at 5 °C. 0.5-ml fractions were collected (from the top). Equal amounts of each fraction were subjected to Western blot analysis. Equal amounts of each fraction were subjected to 10% SDS-PAGE and analyzed by Western blotting. To isolate lipid rafts from larvae, each animal was lysed in 100 μl of TNET buffer and then treated with 1% Triton X-100 through a 27-gauge needle. Debris were removed by centrifuging the lysates at 5000 × g for 5 min. The crude extract was mixed with 200 μl of 60% OptiPrep in TLS55 tubes (Beckman) and then overlaid with 900 μl of 30% OptiPrep, 300 μl of 50% OptiPrep. Gradients were centrifuged at 40,000 rpm at 4 °C for 5 h. 0.5-ml fractions were collected (from the top) and analyzed by Western blotting as described above.

Cholesterol/Sterol Depletion of S2 Cells Using Methyl-β-cyclodextrin and Lovastatin—We examined the effects of lovastatin (gift from Merck and Co.) and methyl-β-cyclodextrin at a variety of concentrations, checking both cell viability and cholesterol/ergosterol levels to identify...
the optimal conditions and drug concentrations. Confluent S2-DWnt-1 cells were incubated in lipid-free medium (Schneider’s *Drosophila* medium with 5% charcoal-stripped fetal bovine serum and 2.5% penicillin/streptomycin) containing 0.5% methyl β-cyclodextrin (Sigma) with ethanol or with 100, 200, and 500 μM lovastatin (Calbiochem) for 2–3 h at 25 °C and then heat-shocked at 37 °C for 1 h followed by a 2-h recovery at 25 °C. Cells were monitored hourly for viability. Loss on cell viability was ~5% for cells treated with 100 and 200 μM lovastatin and 20–30% for cells cultured in 500 μM lovastatin. After treatment, the cells were collected and washed. Whole cell lysates were prepared and analyzed by fractionation over butyl-Sepharose as described above. Total lipids were extracted as described in Ref. 26. Neutral lipids were analyzed by multi-one-dimensional thin layer chromatography as described previously (27) using three sequential solvent systems: chloroform:methanol:acetic acid (90:10:0, v/v/v) and then hexane:diethyl ether:etanol (60:40:5, v/v/v) followed by hexane:ethyl ether (97.3, v/v). The thin layer chromatography plates were sprayed with Premulin dye (Sigma), and the fractionated lipids were visualized using a PhosphorImager Storm 840 imaging system (Amersham Biosciences). Each lipid spot was compared with known standards and quantified using ImageQuant 2.0 software (Amersham Biosciences). After growth in 0.5% methyl-β-cyclodextrin and 100 μM lovastatin, S2 cells showed a 62% decrease in total cholesterol/ergosterol levels.

2-Bromopalmitate Treatment of S2 Cells—S2-DWnt-1 cells were grown to confluency and transferred into low fetal bovine serum medium (Schneider’s *Drosophila* medium with 2.5% fetal bovine serum and 0.5% penicillin/streptomycin). Cells were incubated for 1–2 h at 25 °C with 2-bromopalmitate ranging from 0, 200, 400, and 800 μM in MeSO. DWnt-1 expression was induced by heat shocking cells at 37 °C for 1 h and allowing them to recover at 25 °C for 2 h. Preparation of conditioned medium was as described in Reichsman et al. (4). Protein concentrations were determined using the BCA assay (Pierce).

**RESULTS**

**A Posttranslational Modification in the ER Converts DWnt-1 Into a Membrane-anchored Protein**—We started these studies by asking whether DWnt-1 displays hydrophobic properties similar to those described for murine Wnt-3a (5). The first set of experiments examined the hydrophobicity of Wnt-1 using standard hydrophobic chromatography techniques (Fig. 1a). Whole cell lysates were prepared from transgenic S2 cell lines expressing Wnt-1 under control of the inducible Hsp70 promoter (S2-DWnt-1). Extracts were solubilized in the presence of 0.025% CHAPS and then incubated with butyl-Sepharose. After incubation, the hydrophobic resin was washed, unbound proteins were collected, and bound proteins were eluted in a series of salt and detergent washes. All fractions were then analyzed by Western blotting. Hydrophobic proteins typically do not bind butyl-Sepharose; peripheral membrane proteins bind weakly and are usually eluted by agents that disrupt electrostatic and H-bond interactions. Integral membrane proteins bind tightly and are eluted only by detergents and organic solvents. The three panels in Fig. 1a show the elution profiles for DWnt-1, PCNA (a soluble hydrophilic nuclear protein), and DFz2 (a seven-pass transmembrane protein). As expected, PCNA did not bind butyl-Sepharose and was recovered in the flow-through (Fig. 1a, middle panel). DWnt-1 bound to the butyl-Sepharose beads, remained bound in subsequent low ionic strength washes, and was finally eluted by 1% SDS, a strongly ionic detergent (Fig. 1a, top panel). This type of chromatographic behavior is indicative of proteins that contain a strongly hydrophobic surface. In fact, the elution profile of DWnt-1 was identical to that of DFz2 (compare the top and bottom panels of Fig. 1a).

These observations led us to hypothesize that DWnt-1 is partitioning as a membrane-anchored protein. We tested this hypothesis by asking whether DWnt-1 meets the standard experimental criteria for integral membrane proteins as defined by their behavior during sodium carbonate extraction and Triton X-114 phase separation (Fig. 1, b and c). S2 whole cell lysates were extracted with sodium carbonate, integral mem-

brane proteins were separated from luminal and peripheral membrane proteins by centrifugation, and the fractions were analyzed by Western blotting (Fig. 1b). As expected, PCNA was found in the supernatant, whereas DWnt-1 and DFz2 remained with the membrane fraction. To be certain that DWnt-1 was partitioning into membranes and not simply precipitating at a high pH level, we also carried out Triton X-114 phase separation studies (Fig. 1c). Cells were lysed in the presence of Triton X-114, the insoluble material was discarded, and the aqueous and detergent phases were separated by centrifugation. Both phases were then examined by Western blotting. Fig. 1c shows that PCNA remained in the aqueous phase after Triton X-114 extraction, whereas DWnt-1 and DFz2 partitioned into the detergent phase. Thus in all three experiments, DWnt-1 displayed the characteristic properties of a membrane-anchored protein. This hydrophobicity is remarkably similar to that described previously for murine Wnt-3a (5), suggesting that it may be a feature common to many if not all Wnt ligands.

Two observations suggested that DWnt-1 is lipidated in the ER and that this posttranslational modification creates a membrane anchor. First, we found that DWnt-1 lost its hydrophobic properties after treatment with hydroxylamine-HCl (Fig. 2a). Hydroxylamine-HCl cleaves thioester and ester bonds, releasing fatty acyl groups from cysteine, serine, and threonine residues. Fig. 2a shows the butyl-Sepharose chromatographic profiles for DWnt-1 before and after hydroxylamine treatment. After treatment, the ligand no longer bound butyl-Sepharose, eluting instead in the flow-through fraction. Second, while carrying out a series of *in vitro* translation reactions with and
Without microsomes, we found that untranslocated DWnt-1 remained water-soluble, whereas DWnt-1 synthesized inside the ER partitioned as a membrane-anchored protein after sodium carbonate extraction (data not shown). Together, these results support the hypothesis that Wnt-1 hydrophobicity is a result of lipid modification, and the lipidation process occurs in the ER. Given previous work demonstrating that murine Wnt-3a is palmitoylated at amino acid 77, a conserved cysteine, we tested this idea directly.

2-Bromopalmitate Inhibits DWnt-1 Lipidation—If DWnt-1 is modified by the addition of an ester- or thioester-linked acyl group, it should be possible to block DWnt-1 lipidation by inhibiting acyltransferase activity. We tested this idea by expressing DWnt-1 in the presence and absence of 2-bromopalmitate and then assaying for DWnt-1 hydrophobicity. 2-Bromopalmitate blocks the transfer of palmitate from palmitoyl-CoA to cysteinytl thioles and is a general inhibitor of O-acyltransferase activities (28). The results are shown in Fig. 2b. DWnt-1 protein synthesized in the presence of 400 or 800 μM of 2-bromopalmitate showed a dramatic decrease in butyl-Sepharose binding and an apparent loss of hydrophobicity (Fig. 2b). These results argue that DWnt-1 is lipidated and that its hydrophobicity is most likely attributed to acyltransferase activity. We speculate that the lipid group is likely to be a palmitoyl adduct as has been reported for murine Wnt-3a (5). However, 2-bromopalmitate can also inhibit other enzymes associated with lipid metabolism (29). We have not been able to directly label DWnt-1 using a radiolabeled palmitate or palmitoyl-CoA precursor. Thus definitive confirmation of the lipid moiety may require direct physical measurements such as mass spectrometry analysis of fragmentation patterns.

Lipidation Targets DWnt-1 to Lipid Raft Microdomains—What is the functional significance of DWnt-1 lipidation?

Because many palmitoylated proteins are associated with raft DRMs (6, 7), we wondered whether the lipid modification targets DWnt-1 to these specialized lipid microdomains. We investigated this possibility by isolating raft DRMs from S2-DWnt-1 cells and then assaying for the presence of raft DRM-associated DWnt-1 (Fig. 3). Raft DRMs were isolated by treating S2-DWnt-1 cell lysates with cold 1% Triton X-100, layering the extracts on the bottom of discontinuous iodixamol (OptiPrep) density gradients, and subjecting the gradients to ultracentrifugation. The gradients were then fractionated and analyzed by immunoblotting with specific antibodies. The Western blots in Fig. 3A show the density profiles. We examined DWnt-1, syntaxin 1A (Syx1A), a raft-associated transmembrane protein, Drac1, an isoprenylated protein, and two soluble proteins, Hsp70 and PCNA. Because we used whole cell lysates rather than plasma membrane fractions, we were able to examine the total cellular distri...
bution of each protein. Raft DRMs and raft-associated proteins are expected to float to the less dense fractions at the top of the gradient near the original 5 and 30% iodixanol gradients interface. We verified that we had successfully isolated raft DRMs by examining the density profile for Syx1A, (Fig. 3A, second panel). Syx1A separated into two pools, and most of the Syx1A was soluble and remained in fractions 8–10. However, some Syx1A was raft DRM-associated; this pool floated upward into fractions 2 and 3. The Syx1A profiles we obtained for Drosophila S2 cells were very similar to those reported previously for mammalian Syx1A (30). Hsp70 and PCNA, which are not raft DRM-associated, remained in the dense fractions at the bottom of the gradient (Fig. 3A, bottom two panels, fractions 8–10) after detergent solubilization and centrifugation. We also examined Drosophila Rac-1 (Drac-1). This small GTPase is a useful control because the protein is isoprenylated. Previous studies have shown that despite its lipid modification, isoprenylated Drac-1 does not segregate with lipid raft microdomains (24). Fig. 3A shows that as expected, Drac-1 remained in the soluble fractions and did not float upwards. Thus not all lipid modified proteins are able to partition into the detergent-insoluble fractions.

DWnt-1 behaved very much like Syx1A. Most DWnt-1 remained in the soluble protein fractions; however, some floated to the less dense raft DRMs fractions (Fig. 3A, top panel). In other words, a portion of the total intracellular DWnt-1 was associated with raft DRMs. The fact that most DWnt-1 was detergent-soluble is consistent with earlier studies showing that only a small fraction of DWnt-1 is actually secreted by S2 cells (4). Few cell lines are able to secrete Wnt ligands, and in those that do secretion is very inefficient (31). Assembly of small raft DRMs typically begins in the trans Golgi with larger microdomains forming at the plasma membrane (8). We speculate that most of the lipidated DWnt-1 remains in the ER and/or Golgi compartments.

To be certain that DWnt-1 floated to the less dense fractions only when associated with raft DRMs and not simply because of the posttranslational modification, we repeated these experiments under conditions that prohibit raft DRM assembly. Cholesterol and similar sterols are major constituents of lipid rafts. Drugs such as methyl-br-cyclodextrin and lovastatin deplete cholesterol levels and prevent raft DRM formation (32). We cultured S2-DWnt-1 cells in the presence of 0.5% br-cyclodextrin plus lovastatin, induced DWnt-1 expression, and then assayed for changes in DWnt-1 trafficking. Total neutral lipids were extracted from the treated and non-treated cells and then analyzed by thin layer chromatography to confirm that methyl-br-cyclodextrin and lovastatin treatment depleted the intracellular cholesterol/sterol pools. We found that S2 cells are unusually refractive to treatment with a variety of drugs. In this case, we examined the effects of lovastatin and cyclodextrin at a variety of concentrations, checking both cell viability and cholesterol/ergosterol levels to identify the optimal conditions. When grown in the presence of lipid-free medium treated with 0.5% cyclodextrin and lovastatin for 5–6 h, S2 cells showed a 62% decrease in cholesterol/ergosterol levels. Cells were also monitored for viability. At 100 and 200 μM lovastatin >95% of the cells remained viable, and at 500 μM lovastatin 70–80% of the cells remained viable during the culture period.

Growth in br-cyclodextrin and lovastatin did not affect DWnt-1 hydrophobicity. Even when synthesized in the presence of 200 μM lovastatin, DWnt-1 retained its affinity for butyl-Sepharose binding (Fig. 3B) and continued to partition as a membrane-anchored protein after sodium carbonate extraction (Fig. 3C). We interpret these results to mean that lowering the intracellular cholesterol/sterol levels did not disrupt DWnt-1 acylation. br-Cyclodextrin and lovastatin treatment did inhibit the assembly of raft DRMs in the S2 cells. After lysing treated and untreated cells with cold Triton X-100, we carried out density gradient centrifugations and assayed for raft DRMs as described above. Western blots of the iodixanol gradients are shown in Fig. 3D. As predicted, cholesterol/sterol depletion blocked the formation of Syx1A-associated raft DRMs. When cells were grown in 100 μM lovastatin, we observed a significant decrease in the amount of raft DRM-associated Syx1A. At 200 μM lovastatin, we were unable to detect any Syx1A in the less dense fractions. Cholesterol/sterol depletion also blocked the partition of DWnt-1 into lipid rafts. As the concentration of lovastatin increased, the amount of DWnt-1 floating in the less dense fractions decreased. At 200 μM lovastatin, essentially all DWnt-1 remained in the soluble fractions (fractions 8–10). Together, the experiments shown in Fig. 3 confirm that DWnt-1 is selectively partitioned into raft DRMs.

2-Bromopalmitate Inhibits DWnt-1 Association with Raft DRMs—Palmitate and other long chain saturated fatty acids often partition with cholesterol and glycosphingolipids (7, 33). This led us to wonder whether the lipid group attached to DWnt-1 group is responsible for partitioning the ligand into lipid raft DRMs. We investigated this possibility by asking whether 2-bromopalmitate treatment can inhibit the assembly of DWnt-1-associated raft DRMs. DWnt-1 expression was induced in the presence and absence of 2-bromopalmitate, the cells were collected, treated with cold Triton X-100, and raft DRMs were isolated by discontinuous density gradient centrifugation. Western blots of the gradient fractions are shown in Fig. 4. Again Syx1A was used as a positive control for raft-targeted proteins. Because Syx1A is not palmitoylated, inhibiting O-acyltransferase activity should not affect the association of Syx1A with raft DRM. The blots in Fig. 4 confirmed this. Even when the cells were grown in the presence of 800 μM 2-bromopalmitate, Syx1A continued to partition into raft DRMs. The results obtained for DWnt-1 were quite different in that 2-bromopalmitate inhibited DWnt-1 incorporation into the lipid rafts, and the inhibiting effect appeared to be concentration dependent. When cells were cultured in 200 μM 2-bromopalmitate, the amount of DWnt-1 that floated to the raft fractions was decreased. The pool of raft DRM-associated DWnt-1 continued to drop at 400 and 800 μM inhibitor. These data indicate that O-acyltransferase activity is essential for DWnt-1 targeting to lipid raft DRMs. Together with the findings described above, the results argue that DWnt-1 acylation is a requisite step for lateral targeting of the ligand to specialized detergent-resistant regions within the membrane.

Porcupine, a Putative O-Acyltransferase, Is Required for DWnt-1 Hydrophobicity and for Raft Targeting—Next, we
control animals partitioned with intrinsic membrane proteins during both assays. However, DWnt-1 obtained from *porc* animals, *i.e.* DWnt-1 synthesized in the absence of Porcupine, fractionated with soluble hydrophilic proteins. When *porc* lysates were treated with sodium carbonate, DWnt-1 was released into the supernatant (Fig. 5B). Further quantification analysis by ImageJ showed that in *porc* animals, only 0.68% of DWnt-1 protein was membrane-associated versus 80.76% of DWnt-1 in wild type animals. Similarly, when *porc* lysates were extracted with Triton X-114, DWnt-1 partitioned into the soluble aqueous phase (Fig. 5C). We conclude that porcupine is required for DWnt-1 to exhibit its normal hydrophobicity and membrane association.

We also examined DWnt-1 targeting to raft DRMs in the presence and absence of Porcupine. We isolated individual control and *porc* larvae, solubilized the tissues with cold Triton X-100, and isolated raft DRMs by discontinuous density gradient ultracentrifugation. Western blots of the gradient fractions are shown in Fig. 5D. The *top four panels* show the profiles obtained for the control animals. DWnt-1 and Syx1A were present in both Triton X-100-soluble and detergent-insoluble fractions, whereas Drac1 and Hsp70 remained in the dense fractions at the bottom of the tube. These results demonstrate for the first time that DWnt-1 is targeted to raft DRMs in larval tissues as well as cultured S2 cells.

When we analyzed DWnt-1 obtained from *porc* animals, we obtained very different results. The entire pool of DWnt-1 synthesized in *porc* animals remained in the detergent-soluble fractions (Fig. 5D). We did not find any DWnt-1 floating in the less dense raft DRMs fractions. However, the association of Syx1A was not disrupted in the *porc* animals, indicating that *porc* animals are able to assemble lipid raft microdomains and that other proteins can be partitioned into raft DRMs in the absence of Porcupine. We conclude that Porcupine activity is essential for the lateral targeting of DWnt-1 to raft DRMs in vivo. These findings lead us to hypothesize that DWnt-1 targeting to raft DRMs may be a requisite step for normal DWnt-1 secretion.

In summary, we have found that Porcupine is required for DWnt-1 hydrophobicity and raft targeting, and previous studies have shown that Porcupine is also required for DWnt-1 secretion. Can all Porcupine-dependent defects in DWnt-1 signaling be attributed to the loss of DWnt-1 acylation? One way to address this question is to ask whether all of the Porcupine defects can be phenocopied by inhibiting O-acyltransferase activity. We have already shown that 2-bromopalmitate treatment of S2 cells is sufficient to inhibit DWnt-1 hydrophobicity and raft targeting. In a last set of experiments, we asked whether the inhibitor also blocks DWnt-1 secretion.

S2-DWnt-1 cells were washed, heat-shocked, and cultured for 3 h with or without 2-bromopalmitate. Both conditioned media and cells were collected and analyzed by Western blotting. The results are shown in Fig. 6. Whole cell lysates prepared from untreated and treated cells contained equivalent amounts of DWnt-1 and Hsp70 (Fig. 6), indicating that 2-bromopalmitate treatment did not inhibit gene expression during the 3-h recovery. Likewise, the two conditioned media samples contained equivalent amounts of total extracellular protein (1.61 μg/μl in the untreated sample versus 1.65 μg/μl in the treated sample) demonstrating that 2-bromopalmitate treatment did not act as a general inhibitor of protein secretion. However, secreted DWnt-1 was present only in the untreated conditioned medium (compare the *two top panels* in Fig. 6). When DWnt-1 was synthesized in the presence of the O-acyltransferase inhibitor, we were unable to detect any extracellular ligand. Either DWnt-1 was not secreted or it was rapidly degraded. In either case, DWnt-1 was not targeted to raft DRMs.
case, the absence of detectable extracellular DWnt-1 correlates with immunocytochemical studies showing the absence of extracellular DWnt-1 staining in porc mutant animals (21, 36). Together, these experiments show that 2-bromopalmitate treatment was able to phenocopy each of the known Porcupine-dependent defects, suggesting again that maturation and secretion of active DWnt-1 ligand requires an acyltransferase activity encoded by porcupine. We have also found that blocking raft DRM formation by cholesterol/sterol depletion also blocked the secretion of DWnt-1 but not that of Hsp70 (data not shown). Although we cannot rule out the possibility that blocking sterol synthesis may have a general inhibitory effect on protein secretory pathways in the cells, these observations support the idea that appropriate sorting of DWnt-1 (into lipid rafts) is critical for its secretion.

**DISCUSSION**

Nusse and co-workers (5) first demonstrated that murine Wnt-3a is palmitoylated at a conserved cysteine residue and proposed that palmitoyl modifications may be a common feature among Wnt ligands. Our studies provide evidence that Drosophila Wnt-1 is also lipidated. We have shown that newly synthesized DWnt-1 undergoes a posttranslational modification in which a cholesteryl-linked lipid group is attached to the peptide backbone. Attachment of the lipid affects subsequent DWnt-1 trafficking and secretion in that the lipidated ligand partitions as a membrane-anchored protein and is targeted to lipid raft DRMs before secretion. This lipid modification requires Porcupine, a putative ER membrane-bound O-acyltransferase and can be inhibited by 2-bromopalmitate, a competitive inhibitor of palmitoyl acyltransferases.

Lipidation is likely to play a significant role in the maturation and secretion of many if not all Wnt ligands. Recent studies with mammalian cells have shown that Porcupine is required for secretion of murine Wnt-1, -3A, -4, -6 and -7B (18). Interestingly, whereas vertebrates have a single porc gene, four different porc transcripts are generated via alternative mRNA splicing (18, 19). Sequence analyses suggest that each transcript encodes a putative acyltransferase; however, it is possible that the isoforms exhibit different lipid and protein substrate specificities (35).

**How important is Wnt lipidation? What is its function?** Here we have focused on the upstream events that control production and proper secretion of active DWnt-1 ligand. Animals lacking porcupine activity synthesize DWnt-1, but the unmodified protein does not associate with membranes and is not secreted. Both defects can be phenocopied in S2 cells by the addition of 2-bromopalmitate. These findings are consistent with a growing body of literature demonstrating that lipidation is often used to regulate protein trafficking. Cooperative lipid-lipid interactions between the lipid moieties and membrane lipids can help target the modified protein to specific vesicles and lipid microdomains. Palmitate groups, which partition with cholesterol and sphingolipids, are important determinants for apical sorting and targeting to raft DRMs (8). We have found that the membrane-associated DWnt-1 is partitioned into lipid raft DRMs. Lipidation appears to be requisite for raft targeting as the association of DWnt-1 with raft DRM particles is dependent on porcupine activity and can be blocked by treating cells with 2-bromopalmitate. Our experiments do not rule out the possibility that DWnt-1 undergoes multiple lipid modifications. Additional palmitate groups or other lipid moieties may be added in subsequent steps. Many proteins require at least two saturated acyl chains for raft targeting (36).

Based on our results, we propose a pathway for DWnt-1 maturation and trafficking to the cell surface as follows. DWnt-1 undergoes at least two types of modifications in the ER, which are a Porcupine-dependent fatty acylation and the addition of two Asn-linked high mannose sugars. After glycosylation and lipidation, the protein is transported from the ER to the Golgi where it is partitioned into polarized vesicles and then transported to cholesterol/sphingolipid-rich raft DRMs at the cell surface. In porc mutant animals, DWnt-1 is synthesized and glycosylated, but in the absence of functional Porcupine the ligand is not lipidated. Lacking the appropriate fatty acid sorting signal DWnt-1 does not partition with cholesterol/sphingolipid-rich microdomains and is not transported to the cell surface. Immunocytochemical staining of DWnt-1 expression in porcine mutant animals suggests that the immature ligand accumulates in the ER and/or early Golgi vesicles (34). Similar results have been reported for apolipoprotein B. Palmitoylation of apolipoprotein B concentrates the protein in specialized compartments within the ER, stimulates ER to Golgi transport, and promotes secretion (37).

In summary, our data argue that DWnt-1 is not secreted through the classical constitutive pathway. Instead, DWnt-1 undergoes a Porcupine-dependent lipidation; the lipid group functions as a sorting signal, targeting the ligand to polarized vesicles that transport DWnt-1 to unique sites at the cell surface. This pathway is not unlike apical secretion in mammalian cells, where secretory proteins partition with cholesterol-sphingolipid rich microdomains within the trans Golgi network and are then transported directly to the plasma membrane (8). After reaching the cell surface, how is DWnt-1 secreted? Numerous immunocytochemical studies indicate that extracellular DWnt-1 is concentrated in large punctate particles (38, 39). Given that extracellular Wnts are lipid modified (Ref. 5),2 secretion is likely to require additional processing steps in which the ligand is either packaged into extracellular secretory vesicles or assembled into lipoprotein aggregates that are then released from the cell surface. The first model is compatible with work by Greco et al. (40) suggesting that extracellular DWnt-1 is transported via argosomes, extracellular vesicles that travel from cell to cell. We note that argosome budding and release may take place near lipid raft DRMs (40, 41). If so, then Porcupine-dependent acylation and subsequent raft targeting may ensure that DWnt-1 is concentrated at the site of argosome budding, perhaps promoting the subsequent packaging of DWnt-1 into argosome vesicles.

Our data, considered with other recent studies on Wnt and Hh signaling, suggest that Wnt and Hh ligands may utilize similar mechanisms for transport, secretion, and extracellular...
movement (14). Hh contains both palmitoyl and cholesterol groups; these lipid modifications target Hh to lipid raft DRMs at the surface (13, 24). Extracellular Hh-Np is also found in large particles. The assembly of these Hh-Np aggregates depends on cholesterol and dispatched, and movement of the aggregates from the signaling to the receiving cells is dependent on heparan sulfate proteoglycans (13, 42–44). In summary, lipid modifications provide important signals for targeting morphogens to specific membrane compartments, and they are likely to participate in the packaging, secretion, and perhaps delivery of the morphogen to the responding cells.

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