An in vitro fatty acylation assay reveals a mechanism for Wnt recognition by the acyltransferase Porcupine

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Wnt proteins are a family of secreted signaling proteins that play key roles in regulating cell proliferation in both embryonic and adult tissues. Production of active Wnt depends on attachment of palmitoleate, a monounsaturated fatty acid, to a conserved serine by the enzyme Porcupine (PORCN). Studies of PORCN activity relied on cell-based fatty acylation and signaling assays as no direct enzyme assay had yet been developed. Here, we present the first in vitro assay that accurately recapitulates PORCN-mediated fatty acylation of a Wnt substrate. The critical feature is the use of a double disulfide-bonded Wnt peptide that mimics the two-dimensional structure surrounding the Wnt acylation site. PORCN-mediated Wnt acylation was abolished when the Wnt peptide was treated with DTT, and did not occur with a linear (non-disulfide-bonded) peptide, or when the double disulfide-bonded Wnt peptide contained Ala substituted for the Ser acylation site. We exploited this in vitro assay to provide direct evidence that the small molecule LGK974, which is in clinical trials for managing Wnt-driven tumors, is a bona fide PORCN inhibitor whose IC50 for inhibition of Wnt fatty acylation in vitro closely matches that for inhibition of Wnt signaling. Side-by-side comparison of PORCN and Hedgehog acyltransferase (HHAT), two enzymes that attach 16-carbon fatty acids to secreted proteins, revealed that neither enzyme will accept the other’s fatty acyl-CoA or peptide substrates. These findings illustrate the unique enzyme–substrate selectivity exhibited by members of the membrane-bound O-acyl transferase family.

Wnt proteins comprise a family of secreted signaling molecules that regulate embryonic development as well as tissue homeostasis and tumorigenesis in adults (1). Signaling by Wnt proteins is dependent on covalent attachment of the monounsaturated fatty acid, palmitoleic acid, to a conserved serine residue found in nearly all Wnt family members (Ser209 in Wnt3a) (2, 3). Palmitoleoylation exerts pleiotropic effects on Wnt biology: it is required for Wnt proteins to bind to the chaperone Wntless, for Wnt protein secretion, and for Wnt binding to the Wnt co-receptor Frizzled (4–6). Studies of Wnt fatty acylation in cell-based labeling assays revealed that lipid modification of Wnt proteins occurs in two steps. Stearoyl-CoA desaturase first converts palmitoyl-CoA into palmitoleoyl-CoA, which is then utilized as a substrate by the Wnt acyltransferase Porcupine (PORCN) (7). To date, structural information on PORCN is lacking and Wnt acylation has not been reconstituted in an enzymatic assay in vitro.

PORCN is a member of the membrane-bound O-acyl transferase (MBOAT) family of multipass membrane proteins that catalyze fatty acylation of lipid or protein substrates (8). Three members of the MBOAT family transfer fatty acids to secreted proteins: HHAT, the palmitoyl acyltransferase for Hedgehog proteins; GOAT, which transfers octanoate to ghrelin; and PORCN (9). Each of these fatty acylated proteins contributes to disease states in humans. Because fatty acylation is essential for disease-causing activity, HHAT, GOAT, and PORCN have gained attention as drug targets. Peptide-based fatty acylation assays have led to the development of enzyme inhibitors for HHAT and GOAT (10, 11). Using cell-based Wnt signaling assays, small molecule inhibitors of PORCN have been identified, one of which (LGK974) has progressed to a clinical trial for treatment of Wnt-driven solid tumors (12, 13). However, in the absence of an in vitro assay, the ability of LGK974 to directly inhibit PORCN-mediated Wnt acylation has never been demonstrated. Development of an enzymatic assay for PORCN would shed light on how this enzyme recognizes its substrates and therefore would considerably enhance our knowledge of PORCN-mediated catalysis.

Results

Development of an in vitro assay that recapitulates PORCN-mediated Wnt acylation

We first sought to develop an in vitro assay to directly monitor fatty acylation of Wnt by PORCN. The principle of the assay was based on conditions successfully established for Shh palmitoylation by HHAT (14), with a reaction mix consisting of a biotinylated peptide containing residues surrounding the fatty acylation site, microsomal membranes from cells express-

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2 The abbreviations used are: PORCN, Porcupine; HHAT, Hedgehog acyltransferase; GOAT, ghrelin O-acyltransferase; MBOAT, membrane-bound O-acyl transferase; Shh, Sonic Hedgehog; IC15, iodo-pentadecenoic acid; IC16, iodo-hexadecanoate; FDH, focal dermal hypoplasia.
ing the acyltransferase, and a fatty acyl-CoA substrate. A Wnt peptide was synthesized containing sequences surrounding the Ser\(^{209}\) acylation site of Wnt3a, appended with C-terminal PEG–biotin. This sequence includes the conserved residues required for PORCN-mediated Wnt3a acylation identified in a cell-based acylation assay using \(^{125}\text{I}\)IC15:1 (\(^{125}\text{I}\)iodo-penta-decanoic acid), a radioiodinated palmitoleate analog (15). The presence of the iodide, which is sterically similar to a methyl group, makes the 15-carbon analog behave as a 16-carbon fatty acid. We have previously shown that \(^{125}\text{I}\)IC15:1 is an authentic substrate for PORCN that mimics incorporation of the 16:1 fatty acid into Wnt3a in cells (15). Membranes from PORCN-expressing cells were incubated with the Wnt peptide and \(^{125}\text{I}\)IC15:1 CoA, and biotinylated Wnt peptide was captured on streptavidin–agarose beads. However, no increased radiolabel incorporation into the Wnt peptide incubated with membranes from cells expressing wild-type PORCN was detected when compared with membranes from cells transfected with empty vector (Fig. 1A). We hypothesized that recognition of the serine acylation site, located internally within Wnt, might be different from Shh, where acylation occurs at the N-terminal cysteine. In the three-dimensional crystal structure of Xenopus Wnt8, the acylated serine is located at the base of a loop whose structure is constrained by two sets of disulfide-bonded cysteines (4). We therefore designed a peptide containing amino acids 199–219 of Wnt3a with disulfide bonds between Cys\(^{203}\)–Cys\(^{217}\) and Cys\(^{205}\)–Cys\(^{212}\), and appended with a C-terminal PEG–biotin (WT Wnt (S-S)) (Fig. 1B). As a negative control, a second peptide was synthesized with Ala in place of Ser at the equivalent of position 209. Both peptides were >95% pure based on HPLC analysis. Given the presence of four cysteines, there are three possible arrangements of the two disulfide bonds: Cys\(^{203}\)–Cys\(^{217}\) and Cys\(^{205}\)–Cys\(^{212}\), Cys\(^{203}\)–Cys\(^{205}\) and Cys\(^{212}\)–Cys\(^{217}\), and Cys\(^{203}\)–Cys\(^{212}\) and Cys\(^{205}\)–Cys\(^{217}\) (Fig. 1B). To verify that the disulfide bonds were present in the correct positions, the peptides were digested with trypsin and analyzed by LC–MS on an Orbitrap high-resolution mass spectrometer. The measured monoisotopic mass of the intact peptide was within 10 ppm of the theoretical mass, and the measured monoisotopic mass of the tryptic peptides matches within 10 ppm of the theoretical monoisotopic mass (Fig. 1C). Thus, the correct disposition of the two sets of disulfide-bonded cysteines was experimentally verified.

Incubation of the disulfide-bonded peptide WT Wnt (S-S) with \(^{125}\text{I}\)IC15:1 CoA and membranes from cells expressing wild-type PORCN resulted in a 5-fold increase of radiolabel incorporation when compared with the signal obtained with membranes from cells transfected with empty vector (Fig. 1A). No radiolabel incorporation above background was obtained when the disulfide-bonded peptide lacking the acylation site (S209A WNT) was used as a substrate. Moreover, the acylation signal was vastly reduced with membranes containing H341A PORCN, a putative active site PORCN mutant. The WT Wnt (S-S) acylation reaction was time-dependent (Fig. 1D) and salt-sensitive (Fig. 1E). No radiolabel incorporation into WT WNT was observed when free fatty acid, \(^{125}\text{I}\)IC15:1, was used instead of \(^{125}\text{I}\)IC15:1 CoA (Fig. 1F). We next tested the importance of disulfide-bonded cysteines in the peptide substrate. Pretreatment of WT WNT (S-S) peptide with DTT, prior to the addition of the other reaction components, reduced the signal to background levels (Fig. 1G). This loss of activity was not due to an effect of DTT on PORCN, because pretreatment of PORCN-containing membranes with DTT, followed by membrane re-isolation, had no effect on Wnt peptide acylation (Fig. 1G). Taken together, these data strongly suggest that PORCN-mediated Wnt fatty acylation can be accurately recapitulated in vitro and that the reaction is dependent on the presence of disulfide-bonded cysteines surrounding the Wnt acylation site.

**FDH mutations in Porcn exhibit impaired Wnt acylation activity in vitro**

Mutations in the PORCN gene cause focal dermal hypoplasia, an X-linked disorder characterized by multiple congenital abnormalities (16). Several of the reported FDH-associated missense mutations in human PORCN have been analyzed using a cell-based Wnt acylation assay, and have been shown to exhibit decreased acylation activity as well as decreased protein stability (15). To test whether enzymatic activity of PORCN is directly affected by these FDH mutations, we analyzed Wnt peptide acylation in vitro using membranes from cells expressing wild-type or mutant PORCN. The effects on PORCN-mediated Wnt acylation activity in vitro mirrored those obtained with the cell-based Wnt acylation assay: R228C Porcn exhibited activity nearly identical to WT PORCN, whereas the other mutants were compromised (Fig. 2A).

**Direct inhibition of PORCN activity by LGK974**

Wnt signaling plays a key role in multiple tumor types, especially breast and colon cancer (17). Given that Wnt acylation is essential for signaling activity, inhibitors of PORCN have the potential to serve as chemotherapeutics in Wnt-driven cancers. High-throughput screening was used to identify GNF-1331, a small molecule that inhibits Wnt secretion in cell-based assays of Wnt signaling and also binds to PORCN-containing membranes (13). Pharmacologic optimization of GNF-1331 resulted in the generation of LGK974, which displaces radio- labeled GNF-1331 from PORCN-containing membranes (IC\(_{50}\) = 1 nm) and inhibits Wnt signaling in a co-culture assay (IC\(_{50}\) = 0.4 nm) (13). LGK974 has therefore been designated a PORCN inhibitor and is currently in Phase I clinical trials. To date, however, LGK974 has never been tested for its ability to directly inhibit the enzymatic activity of PORCN. Using our in vitro assay, dose-dependent inhibition of Wnt peptide acylation by LGK974 occurred with an IC\(_{50}\) = 12.9 nm, which closely mirrors the IC\(_{50}\) = 7.5 nm obtained for LGK974 in a luciferase-based reporter assay of Wnt signaling (Fig. 2, B and C).

**In vitro analyses demonstrate substrate specificity of PORCN and HHAT**

PORCN and HHAT both catalyze attachment of 16-carbon fatty acids (palmitoleate or palmitate, respectively) to secreted signaling proteins, and both enzymes are members of the MBOAT family. We previously showed that the small molecule

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Figure 1. An in vitro assay for PORCN-mediated Wnt fatty acylation. A, 10 μg of P100 membranes from cells expressing the indicated constructs (empty vector, WT PORCN, or PORCN (H341A)) were incubated with 100 μM linear or disulfide-bonded peptides representing WT Wnt (black bars) or Wnt S209A (gray bars), and 167 μM [125I]IC15:1 CoA, in reaction buffer for 1 h at 37 °C. Biotinylated peptides were captured with streptavidin–agarose beads, and [125I]IC15:1 incorporation was determined by γ-counting. The experiment was performed three times in duplicate; error bars indicate S.D. B, amino acid sequence of WT Wnt (S-S) peptide and disulfide bond positions. C, 1 μl of C-terminal biotinylated (Biotin–PEG NovaTag™), disulfide-bonded Wnt3a WT (MHL KC(S-)K C(S-)HG LSG SC(S-)E VKT C(S-)WW) diluted 1:50 in 0.1% formic acid or diluted in 1:25 in ammonium bicarbonate was incubated with trypsin (1:100 E:S ratio) for 4 h at 37 °C and analyzed by high-resolution LC–MS over a mass range of m/z 300 – 2000. Shown is zoomed in mass range for the (M + 4H)4 ions at m/z 715 – 720 of the intact peptide and the (M + 2H)2 ions of the two trypsin digestion products at m/z 558 – 562 and m/z 634 – 640. Calcd, calculated. D–G, P100 membranes from cells expressing WT PORCN were incubated with 100 μM WT Wnt (S-S) peptide and [125I]IC15:1 CoA for the indicated time points (D) or in the presence of increasing concentrations of NaCl (E). F, the assay was performed as described in A, with either [125I]IC15:1 CoA or [125I]IC15:1 free fatty acid. G, the assay was performed as in A, with linear or Wnt (S-S) peptide, with Wnt (S-S) peptide treated with 10 mM DTT, or with membranes (mbs) pretreated with 10 mM DTT. Biotinylated peptides were captured with streptavidin–agarose beads, and [125I]IC15:1 incorporation was determined by γ-counting. Panels D–G represent averages from two to three experiments, each performed in duplicate or triplicate; error bars indicate S.D.
Fatty acylation of Wnt by PORCN in vitro

PORCN selectively utilized \([125\text{I}]\)IC15:1 CoA, but not ferases for Wnt and Shh (Hedgehog) proteins, respectively. This suggests that PORCN and HHAT are dedicated acyltransferases with regard to fatty acid and protein substrates, and that these enzymes side by side, we analyzed the ability of each enzyme to recognize each other’s fatty acid and protein substrates. To directly compare the substrate specificities of the two MBOAT enzymes that both catalyze attachment of 16-carbon acyl-CoA, we designed a radiolabel incorporation assay now makes it possible to directly compare, side by side, the effect of fatty acylation and signaling by Wnt3a (15, 18). Moreover, GFP fusions containing 21- and 31-amino acid sequences from Wnt1 that include the four cysteines incorporate alkyne-labeled palmitate in cell-based acylation assays, whereas an 11-amino acid sequence that lacks the outer cysteine pair does not (19). Using synthetic peptides that encompass the sequence surrounding the Ser acylation site, we show that the mere presence of Cys residues in a Wnt substrate is not sufficient, and that disulfide bonding of Cys residues is critical for PORCN-mediated acylation. It is likely that this unique two-dimensional structure places constraints on the orientation of Ser209 so that it can be correctly presented for binding to and acylation by PORCN. The double disulfide-bonded Wnt peptides were extremely technically challenging to synthesize, and as a result, production was time- and labor-intensive and costly. Nonetheless, the 21-residue WT Wnt (S-S) peptide represents a minimal length PORCN substrate for in vitro assays. Further refinement of the geometric requirements within the Wnt peptide substrate could be directed toward replacement of native disulfides with synthetic bridges or disulfide bond mimetics or the use of cyclized peptides.

LGK974 is currently in clinical trials for safety and efficacy in treating Wnt-driven tumors. Although it is marketed as a PORCN inhibitor, the ability of LGK974 to directly inhibit PORCN-mediated Wnt acylation has never been tested. Thus, it remained formally possible that inhibition of Wnt secretion and signaling that occurred with LGK974 treatment in cells could be due to an effect on one or more of the multiple steps required to produce active Wnt ligand, e.g. inhibition of stearoyl-CoA desaturase or binding to Wntless. Here, we exploit the in vitro Wnt acylation assay to provide the first direct evidence that LGK974 is a bona fide PORCN inhibitor whose IC_{50} for inhibition of Wnt fatty acylation closely matches that for inhibition of Wnt signaling. Future mechanistic studies can now be carried out to determine how LGK974 inhibits PORCN-mediated catalysis.

Understanding the molecular basis for enzyme–substrate recognition and selectivity remains one of the major challenges for MBOAT proteins in general, and PORCN and HHAT in particular (20). The development of an in vitro Wnt acylation assay now makes it possible to directly compare, side by side, two MBOAT enzymes that both catalyze attachment of 16-carbon fatty acids to secreted proteins. Neither enzyme will accept the other’s fatty acyl-CoA or protein (peptide) substrates. These biochemical features explain why targeted disruption of PORCN is not genetically rescued by HHAT, and vice versa. A two-dimensional transmembrane topology map for HHAT reveals the presence of 10 transmembrane domains and 2 reentrant loops (21, 22). Although a similar topological analysis of PORCN is not yet available, it is likely to be equally complex. The next step toward understanding how these multipass
membrane proteins function will likely require three-dimensional structural information.

**Experimental procedures**

**Plasmids, cell culture, transfection, and membrane preparation**

N-terminal FLAG-tagged Porcn cDNA was generated from a murine Porcupine clone in pcDNA3.1 (a gift from Dr. Joseph Goldstein (UT Southwestern)). Porcn point mutants were generated by site-directed mutagenesis using the QuikChange mutagenesis kit (Agilent Technologies). Super TOP/FOP and pRL-TK plasmids were a kind gift from Dr. Anthony M. C. Brown (Weill Cornell Medical College, New York, NY). HEK 293FT cells (Invitrogen) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, 500 μg/ml Geneticin, 1 mM GlutaMAX (Invitrogen), 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. Cells were transfected with 6 μg of Porcn cDNA using Lipofectamine (Invitrogen), split 1:2 the following day, and then grown for 24 h. P100 membranes were prepared from hypotonically lysed cells, following centrifugation at 100,000 × g (P100) as described (14).
**Substrates for in vitro fatty acylation assays**

IC15:1 was synthesized by the Organic Synthesis Core Lab of Memorial Sloan Kettering Cancer Center, and converted into [125I]IC15:1 using 125I-NaI (PerkinElmer). Synthesis of [125I]IC15:1 CoA using acyl-coenzyme A synthetase (Sigma catalog number A3352) was carried out as described previously (14). IC16, [125I]IC16, and [125I]IC16 CoA were generated as described (14). C-terminal biotinylated Shh peptide (CGP-GRFGKR) was prepared as described (14). Linear Wnt3a WT (CKCHGLSGCSEFKTCW; calculated mass 2152.984, observed mass 2169.043) and Wnt3a S209A (CKCHLAG-SCEVKTCW; calculated mass 2152.984, observed mass 2153.248) peptides with C-terminal PEG-biotin were synthesized by the Microchemistry Core Lab of Memorial Sloan Kettering Cancer Center. C-terminal biotinylated (Biotin–PEG NovaTag™), disulfide-bonded Wnt3a WT (MHL KC(S)-K C(S)-HG LSG SC(S)-E VKT C(S)-WWW) (calculated monoisotopic mass (M+H)+ = 2860.3052, observed monoisotopic mass (M+H)+ = 2860.2943), and C-terminal biotinylated (Biotin–PEG NovaTag™), disulfide-bonded Wnt3a S209A (MHL KC(S)-K C(S)-HG LAG SC(S)-E VKT C(S)-WWW) (calculated monoisotopic mass (M+H)+ = 2844.3103, observed monoisotopic mass (M+H)+ = 2844.2959) peptides were synthesized by AnaSpec (Fremont, CA), and then analyzed by HPLC to assess purity to be greater than 95% pure. To confirm the identity and location of the disulfide bonds, a 1-µl aliquot was diluted in 25 µl of 100 mM ammonium bicarbonate and subsequently digested with trypsin at a 1:100 enzyme-to-substrate ratio at 37 °C for 4 h. The trypsin-digested peptides as well as undigested peptides were further diluted 1:50 with 0.1% formic acid. 2-µl aliquots were characterized by high-resolution LC–MS on an Orbitrap mass spectrometer (Thermo Scientific LTQ Orbitrap XL) coupled with a Waters nano-ACQUITY LC via a Proxeon2 nano-electrospray ionization source. Peptides were eluted from a nano-C18 reverse-phase column with a 1–50% binary gradient, 0.1% formic acid (Buffer A) and acetonitrile with 0.1% formic acid (Buffer B), over 90 min at a flow rate of 300 nL/min. MS1 data from m/z 380–2000 were acquired in profile mode at 60,000 resolution.

The C-terminal biotinylated (Biotin–PEG NovaTag™), disulfide-bonded Wnt3a WT peptide mass spectra showed signals for the +3, +4, and +5 charge state with the +4 charge state and observed monoisotopic mass shown (Fig. 1C). As seen in Fig. 1B, there are three possible arrangements of the two disulfide bonds: Cys203–Cys217 and Cys205–Cys212, Cys203–Cys205 and Cys212–Cys217, and Cys203–Cys212 and Cys205–Cys217. We took advantage of the lysine residues within the peptide sequence and the location of the disulfide bonds to experimentally confirm the proper orientation of the disulfide bonds. After trypsin digestion, two distinct products will be generated for the desired Cys203–Cys217 (calculated monoisotopic mass (M+H)+ = 1117.4703) and Cys205–Cys212 (calculated monoisotopic mass (M+H)+ = 1270.5697) linked peptide. The alternate arrangements result in two peptides: one of 528.2936 and one of 2389.0634 (calculated monoisotopic mass). Upon 4 h of trypsin digestion, we observed the disappearance of the substrate and the generation of two peptides, (M+2H)2++ ions at m/z = 559.2391 and (M+2H)2++ ions at m/z = 635.7888 corresponding to the measured monoisotopic mass (M+H)+ = 1117.4763 and (M+H)+ = 1270.5780, respectively (Fig. 1C), both within 10 ppm of the calculated monoisotopic mass. Identical data were acquired for the C-terminal biotinylated (Biotin–PEG NovaTag™), disulfide-bonded Wnt3a S209A (intact peptide calculated monoisotopic mass (M+H)+ = 2844.3103, observed monoisotopic mass (M+H)+ = 2844.2959), trypsin-generated cleavage calculated monoisotopic mass (M+H)+ = 1101.4814, measured monoisotopic mass (M+H)+ = 1101.4737, and calculated monoisotopic mass (M+H)+ = 1270.5683.

**In vitro Wnt and Shh peptide fatty acylation assays**

10 µg of P100 membranes harvested from Porcupine-expressing HEK293FT cells were incubated with 100 µM Wnt peptide and 30 µl of reaction buffer (167 mM MES, pH 6.5, 0.083% Triton X-100, 167 µM [125I]IC15:1 CoA) for 1 h at 37 °C. For Shh assays, 10 µg of P100 membranes harvested from HHAT-expressing HEK293FT cells were incubated with 100 µM Shh peptide, 167 µM [125I]IC16 CoA, and 30 µl of reaction buffer for 1 h at room temperature (14). After incubation, 400 µl of radioimmunoprecipitation assay buffer and 50 µl of streptavidin–agarose beads were added, and the mixture was incubated for 1 h at 4 °C with continuous mixing. Biotinylated peptides were pelleted by centrifugation at 1000 × g for 5 min. Pellets were washed three times with 500 µl of radioimmunoprecipitation assay buffer. [125I]IC15:1 or [125I]IC16 incorporation was measured in a PerkinElmer γ counter.

**Wnt signaling activity assays**

To measure paracrine Wnt signaling activity, HEK293FT cells were transfected with 3 µg of Super TopFlash or Super FOP and 0.3 µg of pRL-TK, and 24 h after transfection, cells were co-cultured in 12-well plates with L-Wnt3a cells transfected with 6 µg of Porcn at a 3:1 ratio (L-Wnt3a:293FT) in the presence of the indicated drugs for 24 h. Wnt3a pathway activity was detected using a Dual-Luciferase reporter assay (Promega) and recorded as relative luciferase units using a BioTek Synergy™ H1 microplate reader.

**Author contributions**—J. J. A. conducted all of the Wnt acylation experiments and analyzed the data. M. M. M. and R. C. H. performed the mass spectrometry analyses of the Wnt peptides. M. D. R. conceived the idea for the project, designed the peptides, and wrote the paper along with the other authors.

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