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Monounsaturated Fatty Acid Modification of Wnt Protein: Its Role in Wnt Secretion

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Summary

The secretion and extracellular transport of Wnt protein are thought to be well-regulated processes. Wnt is known to be acylated with palmitic acid at a conserved cysteine residue (Cys77 in murine Wnt-3a), and this residue appears to be required for the control of extracellular transport. Here, we show that murine Wnt-3a is also acylated at a conserved serine residue (Ser209). Of note, we demonstrated that this residue is modified with a monounsaturated fatty acid, palmitoleic acid. Wnt-3a defective in acylation at Ser209 is not secreted from cells in culture or in *Xenopus* embryos, but it is retained in the endoplasmic reticulum (ER). Furthermore, Porcupine, a protein with structural similarities to membrane-bound O-acyltransferases, is required for Ser209-dependent acylation, as well as for Wnt-3a transport from the ER for secretion. These results strongly suggest that Wnt protein requires a particular lipid modification for proper intracellular transport during the secretory process.

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Introduction

The Wnt family of secreted signal proteins plays a key role in numerous aspects of embryogenesis, as well as in carcinogenesis (Logan and Nusse, 2004; Moon et al., 2004; Reya and Clevers, 2005). Most Wnt proteins transmit signals locally, presumably since their secretion and transport are under tight control. Although the molecular mechanism underlying their secretion and transport remains largely unknown, recent successes in identifying various molecules involved in these processes provide further clues. For instance, Wntless/Evi, a seven-pass membrane protein, plays an essential role in Wnt secretion (Banziger et al., 2006; Bartscherer et al., 2006), and a protein complex involved in intracellular membrane trafficking, called the retromer complex, participates in the long-range transport of Wnt protein (Coudreuse et al., 2006; Prasad and Clark, 2006). In addition, heparan sulfate-modified proteoglycans are involved in Wnt signaling, possibly by regulating the extracellular transport of Wnt (Hacker et al., 2005; Lin, 2004).

One important step regulating the extracellular transport of various secreted signal proteins, including Wnt, Hedgehog (Hh), and Spitz (*Drosophila* Transforming Growth Factor α), involves posttranslational modification with lipid moieties (Mann and Beachy, 2004; Miura et al., 2006; Nusse, 2003). A well-known example is cholesterol modification of the COOH terminus of the active form of Hh, which is generated by auto-proteolytic cleavage and is required for restricting the range of action of this protein (Lewis et al., 2001; Porter et al., 1996a, 1996b). In addition to cholesterol modification, a fatty acid modification, i.e., acylation, occurs with Hh, Wnt, and Spitz (Miura et al., 2006; Pepinsky et al., 1998; Willert et al., 2003). To date, at least three types of acylation are known to occur in eukaryotic cells: N-myristoylation, S-palmitoylation, and N-palmitoylation. N-myristoylation refers to the covalent modification with myristate at the N-glycine of proteins, S-palmitoylation refers to the reversible addition of fatty acids to cysteine residues through thioester linkages, and N-palmitoylation, first described for Hedgehog protein, involves modification at the N-terminal residues of proteins (Linder and Deschenes, 2003; Smotrys and Linder, 2004). In the case of Wnt, Nusse and coworkers reported that murine Wnt-3a is S-palmitoylated at a conserved cysteine residue at the 77th residue (Cys77) (Willert et al., 2003). A mutant form of mouse Wnt-3a, in which the palmitoylated Cys77 is substituted with alanine (C77A), shows a diminished ability to activate Wnt signaling, but is secreted normally into the culture medium (Willert et al., 2003). Thus, the authors proposed that palmitoylation of this cysteine residue may be required to produce an increased local concentration of Wnt on the plasma membrane. However, although their mass spectrometry analysis covered 85% of the primary amino acid sequence of Wnt-3a (Willert et al., 2003), there remains the possibility of additional acylation sites.

There is strong evidence to suggest that acylation is involved in the processing and intracellular trafficking

of Wnt prior to secretion. Genetic evidence suggests that Wnt-secreting cells require the action of specific genes, e.g., *porcupine* (*porc*) in *Drosophila* or its ortholog, *mom1*, in *C. elegans*, both of which encode proteins with structural similarities to those of a family of membrane-bound O-acyltransferases (MBOAT), which transfer acyl groups, such as a palmitoyl group, to substrates (Hofmann, 2000; Kadowaki et al., 1996; Thorpe et al., 1997; van den Heuvel et al., 1993). Porcupine (Porc) is localized at the endoplasmic reticulum (ER), and its overexpression in culture cells enhances the intracellular processing, for example, N-glycosylation, of Wingless (Wg: the *Drosophila* Wnt-1 ortholog) (Tanaka et al., 2002). In addition, treatment with a chemical inhibitor of acyltransferases produces defective intracellular trafficking of Wg (Zhai et al., 2004). Thus, *porc*-dependent acylation may regulate the processing and intracellular trafficking of Wnt, although acylation at Cys77 does not appear to be involved in these processes.

To resolve inconsistencies between studies examining the roles of Wnt acylation, and to better understand the biological significance and molecular mechanism of Wnt acylation, we carefully examined which amino acid residues are acylated, as well as the function of acylation. Our results indicate that another acylation with a monounsaturated fatty acid occurred at a conserved serine, and we suggest that Wnt-3a defective in acylation at this site does not exit the ER.

Results

Ser209 Is Required for Acylation of Wnt-3a, as Determined by Metabolic Labeling

We first examined acylation of Wnt-3a by metabolic labeling of cultured cells. In cultures of Wnt-3a-expressing mouse L cells (Shibamoto et al., 1998), specific labeling of secreted Wnt-3a protein with radiolabeled palmitic acid was observed, as previously reported. This labeling was detected after only 4 hr of incubation (Figure 1A), while labeling with radiolabeled cholesterol was not detected, even after 36 hr of incubation (data not shown). Palmitoyl moieties, when linked by thioester, but not oxyester, bonds, are known to be displaced from proteins by high concentrations of disulfide-reducing agents, such as 2-mercaptoethanol or dithiothreitol (Bizzozero, 1995). However, even though a previous report describes palmitoylation of Wnt-3a through a thioester linkage at Cys77 (Willert et al., 2003), we detected labeling even after incubation with a high concentration of a disulfide-reducing agent, i.e., 1.3 M 2-mercaptoethanol. We also observed that this labeling was resistant to neutral hydroxylamine (pH 7.0), which specifically cleaves thioester linkages (data not shown) (Bizzozero, 1995). These results suggest that acylation occurs at another site of Wnt-3a via an oxyester bond, in addition to the known acylation of Wnt-3a at Cys77 through a thioester linkage. Similarly, radiolabeled Wnt-5a was detected in cultures of Wnt-5a-expressing L cells under the same conditions, indicating that acylation commonly occurs among Wnt family members (Figure 1B). To confirm acylation of Wnt-3a at a site other than Cys77, we examined acylation of a mutant form of Wnt-3a, in which Cys77 was substituted by alanine (C77A). A significant reduction in acylation was not observed in C77A, indicating that another acylation site, besides Cys77, exists in Wnt-3a (Figure 1A).

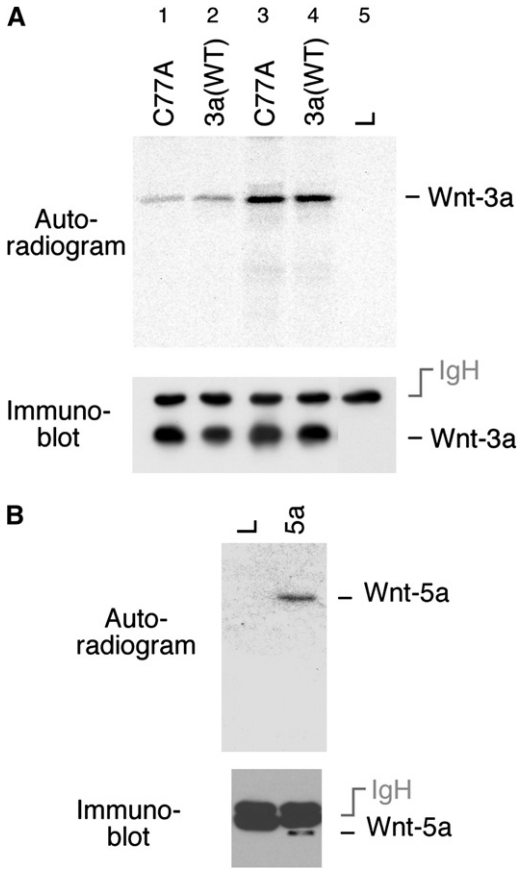

Figure 1. Acylation of Wnt-3a Protein Detected by Metabolic Labeling with Radiolabeled Palmitic Acid

(A) Acylation of wild-type and C77A forms of Wnt-3a proteins secreted from metabolically labeled cells was examined by autoradiography of anti-Wnt-3a immunoprecipitates (upper panel). Wild-type-producing (lanes 2 and 4), C77A-producing (lanes 1 and 3), and control (lane 5) L cells were incubated with ^{14}C-palmitic acid for 4 hr (lanes 1 and 2) or 36 hr (lanes 3–5). Immunoprecipitated Wnt-3a protein from culture supernatant was identified by immunoblotting with anti-Wnt-3a antibody (lower panel). Immunoglobulin heavy chains (IgH) reactive with the secondary antibody were also detected.

(B) Acylation of Wnt-5a protein secreted from Wnt-5a-producing L cells incubated with ^{3}H-palmitic acid for 36 hr was examined by immunoprecipitation by following the same procedure described in (A).

To map out the additional site of acylation within the amino acid sequence of Wnt-3a, we generated C-terminal-truncated forms of Wnt-3a protein and examined acylation (Figure 2A). Whereas Wnt-3a truncated at the 281st residue (ΔC-281) contained radiolabeled palmitic acid upon recovery from the cell lysate, as did the full-length protein (FL), Wnt-3a protein truncated at the 202nd residue (ΔC-202) did not contain the radiolabel (Figure 2B). Of note, in the region extending between the 203rd and 281st residues, numerous amino acid residues, including three serines and a threonine, were found to be highly conserved among members of the Wnt family (Figure 2A). Because serine and threonine may form oxyester linkages with acyl moieties, we next examined whether any of the conserved residues

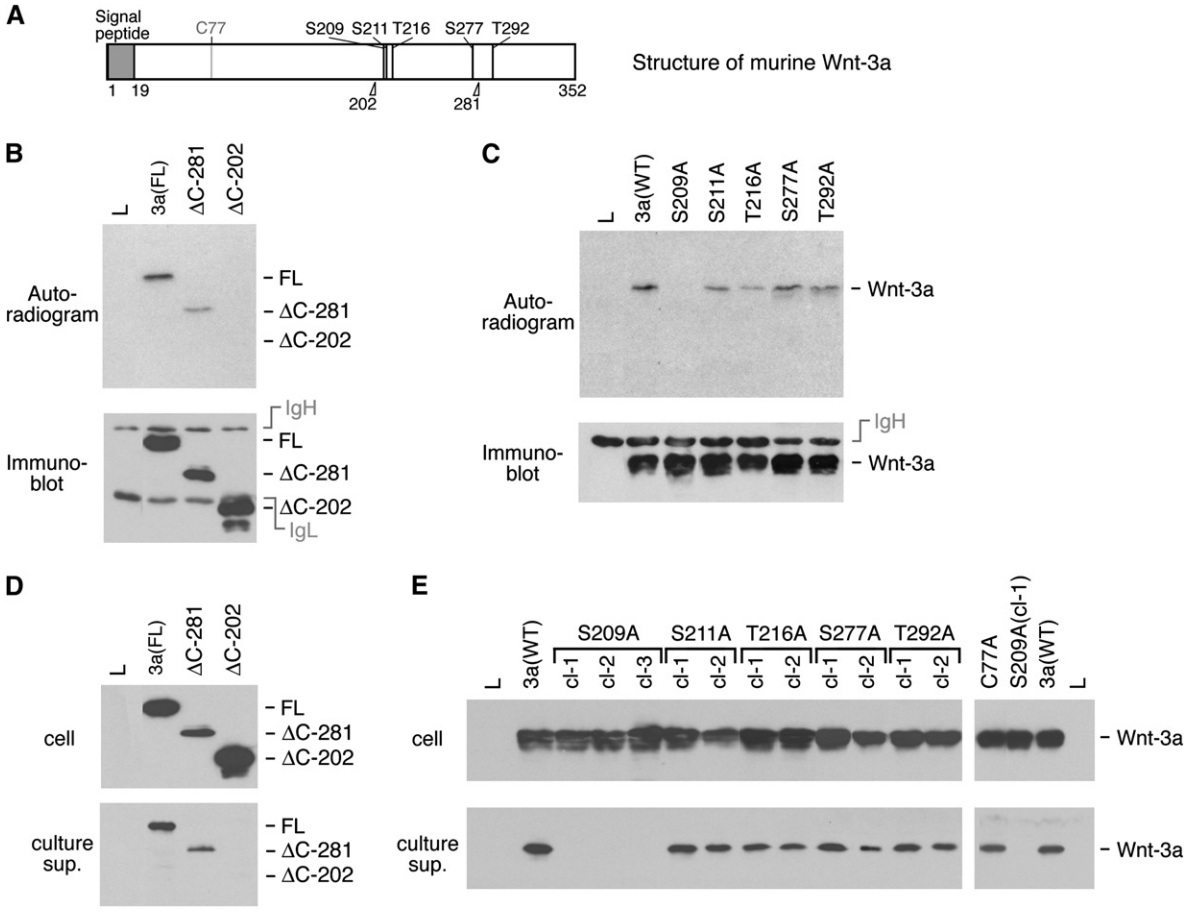

Figure 2. Serine 209 Is Required for the Acylation and Secretion of Wnt-3a in Cultured Cells

(A) Schematic representation of the sites truncated or mutated in this study.

(B) Acylation of truncated forms of Wnt-3a protein was examined by metabolic labeling by following the same procedure described in Figure 1A, except that samples were prepared from cell lysates. Control L cells and L cells expressing the HA-tagged full-length (FL) or one of two truncated (ΔC-281, ΔC-202) forms of Wnt-3a were incubated with ^{3}H-palmitic acid for 36 hr. All immunoprecipitates were obtained from cell lysates by using anti-HA antibody (upper panel). Immunoprecipitation of Wnt-3a protein was confirmed by using anti-HA antibody (lower panel). Coprecipitation of immunoglobulin heavy chains (IgH) and light chains (IgL) was identified.

(C) Acylation of point-mutated forms of Wnt-3a protein was examined as shown in (B) by using anti-Wnt-3a antibody. As controls, analyses of immunoprecipitates from L cells producing wild-type Wnt-3a (3a(WT)) and from control L cells (L) are also shown.

(D) Secretion of the two truncated (ΔC-281, ΔC-202) forms of Wnt-3a protein, as well as the HA-tagged full-length (FL) Wnt-3a protein, into the culture medium was examined. The amount of Wnt-3a protein within cells (upper panel) and released into the culture medium (lower panel) was examined by immunoblotting with anti-HA antibody.

(E) Secretion of the five point-mutated forms of Wnt-3a proteins was examined by using anti-Wnt-3a antibody as shown in (D). For each point-mutated form, more than two independent cell clones (cl) are shown. The representative clones of each form shown in (C) are numbered "cl-1." As controls, secretion of wild-type and C77A forms of Wnt-3a was also examined.

might be required for acylation to occur, by substituting each of them with alanine (S209A, S211A, T216A, and S277A). A threonine-to-alanine mutation outside of this region (T292A) was also included in the analysis as a control. It was remarkable that no acylation was detected for S209A, while acylation of the four other mutated Wnt-3a proteins (S211A, T216A, S277A, and T292A) matched that of wild-type Wnt-3a (Figure 2C). Thus, we concluded that Ser209 was required for detectable acylation of Wnt-3a under these assay conditions. We should also note that despite a previous report indicating that overexpression of *porc*, a putative membrane-bound O-acyltransferase (Hofmann, 2000), enhances Wg glycosylation (Tanaka et al., 2002), no obvious defect in N-glycosylation was detected in the S209A mutant when compared with wild-type Wnt-3a, in which N-glycosylation produces two products of different sizes, both of which are sensitive to tunicamycin treatment (Figure S1; see the Supplemental Data available with this article online).

Wnt-3a Is Modified with a Monounsaturated Fatty Acid at Ser209

Next we investigated whether acylation of Wnt-3a actually occurs at Ser209, as well as the structure of the attached moiety, by mass spectrometry (MS). FLAG-tagged Wnt-3a protein was purified from the conditioned media of the expressing cells (Figure S2), treated with trypsin, and then subjected to nano-flow reverse-phase liquid chromatography (LC) followed by MALDI-MS/MS. A number of peptides obtained after trypsin digestion, including 75% of the amino acid sequence

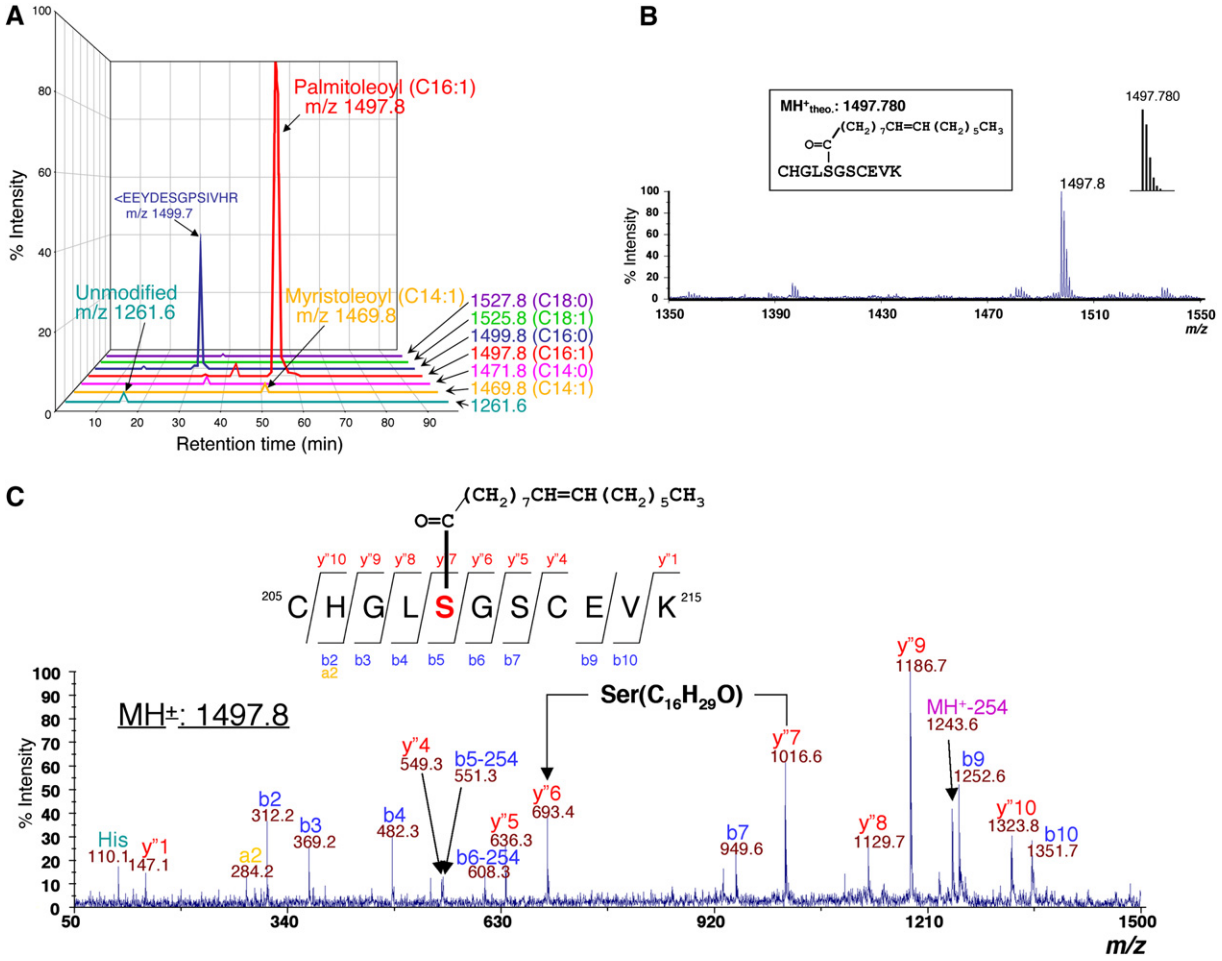

Figure 3. Lipid Modification of Wnt-3a Protein Probed by Nano-Flow LC/MALDI-MS and MS/MS

(A) The selected ion chromatogram reconstituted on the basis of m/z 1261.6, 1469.8, 1471.8, 1497.8, 1499.8, 1525.8, and 1527.8, corresponding to the respective theoretical MH^+ values of unmodified peptide and peptide modified with C14:1-, C14:0-, C16:1-, C16:0-, C18:1-, and C18:0-fatty acids. The ion intensities of each trace were normalized to that of the ion at m/z 1497.8. The peptide (m/z 1499.7) eluted at 32 min turned out to be a peptide (<EEYDESGPSIVHR, where <E is pyroglutamic acid) derived from γ-actin.

(B) The MALDI-MS spectrum of the peptide modified with C16:1-fatty acid. The $MH^+_{theo.}$ value and the theoretical isotopic ion distribution in the spectrum (inset) were calculated from the chemical structure indicated in the box.

(C) The MS/MS spectrum from the ion at m/z 1497.8. The spectrum was interpreted by the software *SeqMS* (see Experimental Procedures). The sequences from the N and C termini and the position of the modified residue were read out based on bm and y"l ions, respectively, where m and l denote the arbitrary positions counted from the N and C termini, which were produced by cleavage of peptide bonds during MS/MS. His, b-254 and MH^+254, respectively, in the spectrum denote the immonium ion of histidine and the fragment ions derived from β elimination of the lipid moiety ($C_{16}H_{29}O$) at Ser209 from the respective b or MH^+ ions.

of the secreted form of Wnt-3a, were recovered by using this procedure (Table S1). Unexpectedly, Ser209-containing peptides with the sequence CHGLS^{209}GSCEVK were not obtained at m/z 1499.8, corresponding to a peptide modified with palmitate (C16:0), nor at m/z values of 1471.8, 1525.8, and 1527.8, corresponding to peptides modified with myristate (C14:0), oleate (C18:1), and stearate (C18:0), respectively (Figure 3A). It should be noted that the peptide prominently observed at m/z 1499.7 turned out to be a trypsin digestion product of γ-actin (42 kDa) on MS/MS analysis. Rather, the Ser209-containing peptides were obtained at m/z 1497.8, 1469.8, and 1261.6, corresponding to the theoretical MH^+ values of the peptides modified with palmitoleic acid (C16:1), myristoleic acid (C14:1), and the unmodified peptide, respectively (Figure 3A). Among the three peptides detected, the one observed at m/z 1497.8 (corresponding to modification with C16:1) was the predominant molecular species (Figures 3A and 3B), although the relative amounts of each peptide were not explicitly determined. MS/MS analysis of the predominant peptide revealed the site of modification to be Ser209, based on the prominent fragment ions, y"6 and y"7, as well as b4 and b5 − 254 (Figure 3C). Differences in the masses of these ions could be assigned to Ser (323.2) modified with a C16:1-fatty acid or dehydro-Ala (69.0), respectively, the latter of which is a product of β elimination of a lipid moiety from modified Ser209 during the MS/MS measurement.

Further characterization of this prominent peptide (at m/z 1497.8) was carried out by accurate mass measurement, which indicated a molecular mass of 1496.776.

Thus, the accurate mass of the modification was calculated to be 236.217. This value suggests a unique elemental composition, $C_{16}H_{28}O_1$ (theoretical mass: 236.214), indicating modification with a monounsaturated C16-fatty acid (C16:1). It is also noteworthy that the isotopic ion distribution observed for the ion at m/z 1497.8 almost completely matched the theoretical one calculated for a peptide modified with a C16:1-fatty acid (see Figure 3B and the inset). In addition, this isotopic ion distribution was partially shifted when deuterium-labeled palmitic acid ($CD_3(CH_2)_{14}COOH$) was added into culture of Wnt-3a-expressing L cells (data not shown), indicating that the deuterium-labeled palmitic acid (C16:0), as well as the radiolabeled one in the experiments described above, was metabolically processed to C16:1-fatty acid, which then bound to the peptide. Finally, to elucidate the position of the double bond within the C16:1-fatty acid moiety, the olefinic double bond of the prominent peptide (m/z 1497.8) was subjected to oxidative cleavage. The original peptide was mostly degraded into its oxidative products via the loss of C_7H_{14} from the C16:1-fatty acid moiety (data not shown), suggesting that the double bond is located at Δ^9, which is identical to the position within biosynthesized palmitoleic acid (C16:1). Based on these lines of evidence, we conclude that Wnt-3a is modified with a monounsaturated fatty acid, palmitoleic acid (C16:1), at Ser209.

Ser209 Is Essential for Secretion of Wnt-3a

Next, we investigated whether Ser209-dependent acylation is involved in the secretion of Wnt-3a. We examined the secretion of the two C-terminal-truncated forms and five point-mutated forms of Wnt-3a proteins from L cells into their culture medium, as well as that of the wild-type and C77A forms of Wnt-3a. Interestingly, acylation was coupled with secretion for all Wnt-3a variants. Full-length (FL) and ΔC-281 forms of Wnt-3a demonstrated similar levels of secretion; however, secretion of the ΔC-202 form into the culture medium was not detected (Figure 2D). Furthermore, among the five point mutants, only S209A was not detected in the medium (Figure 2E).

Turnover of wild-type and S209A forms of Wnt-3a proteins was then examined during cycloheximide-mediated inhibition of de novo protein synthesis. S209A protein was stably retained in cells, while most wild-type Wnt-3a protein was secreted into the medium within 24 hr of treatment with cycloheximide (Figures 5A and 5B). Thus, the S209A mutation, preventing the palmitoleoyl modification, did not affect the stability of Wnt-3a protein, but rather blocked its secretion.

Porcupine Is Required for the Acylation and Secretion of Wnt-3a

The gene *porc*, encoding a putative O-acyltransferase, is thought to be required for the secretion and intracellular transport of Wg in *Drosophila* (Hofmann, 2000; Kadowaki et al., 1996; van den Heuvel et al., 1993; Zhai et al., 2004). Thus, we examined whether *porc* is required for detectable Wnt-3a acylation under the conditions of this study. In a number of independent L cell transfectants, in which expression levels of *porc* were stably reduced by expression of siRNA specific for mouse *porc*, reduced acylation of Wnt-3a was observed, reflecting some residual *porc* expression, while the production and glycosylation of Wnt-3a appeared not to be perturbed (Figures 4A and 4B; data not shown). It is not certain whether the acylation remaining in *porc*-deficient transfectants was actually due to residual Porc activity. However, the possibility that the residual acylation was Cys77 palmitoylation, possibly detected even under this condition, can be excluded because levels of residual acylation were not reduced by substitution of Cys77 with alanine (Figure S3). Thus, *porc* is required for most of the acylation, which is also dependent on Ser209. Furthermore, as observed with S209A, Wnt-3a protein was retained in untreated *porc*-deficient cells and in

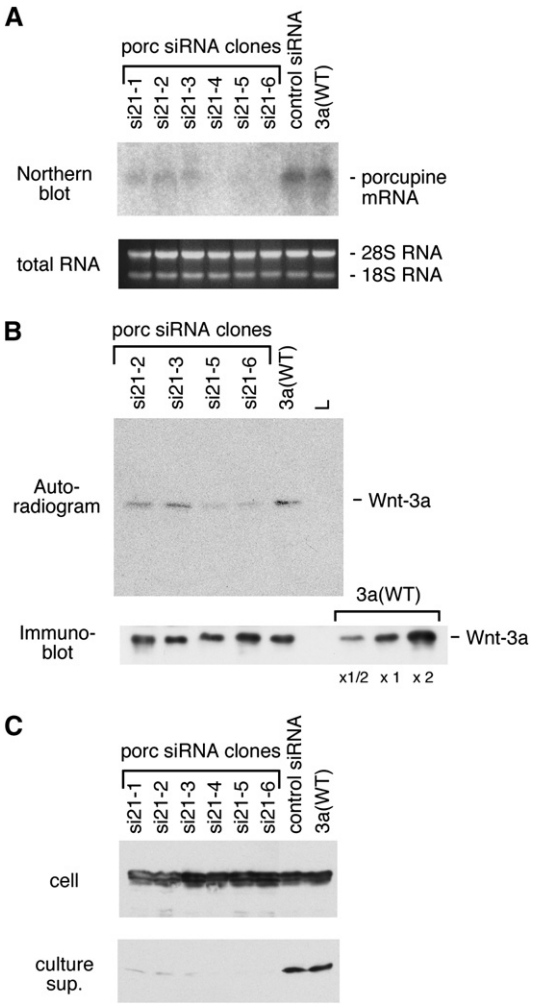

Figure 4. Porcupine Is Required for Palmitoleic Modification and Secretion of Wnt-3a

(A) Endogenous *porc* mRNA expression in clones expressing different levels of *porc*-specific siRNA (si21) is shown (upper panel). As controls, endogenous expression levels in cells expressing control siRNA and in parental Wnt-3a-expressing cells (3a(WT)) are also shown. Total RNAs loaded onto each lane are shown as well (lower panel).

(B) Acylation of Wnt-3a in *porc*-deficient clones, as well as in parental cells expressing Wnt-3a (3a(WT)), and control L cells (L) was analyzed as shown in Figure 2C. Standards to enable quantification of Wnt-3a were also loaded (right).

(C) Reduction in Wnt-3a protein secretion in *porc*-deficient cells was examined as shown in Figure 2E.

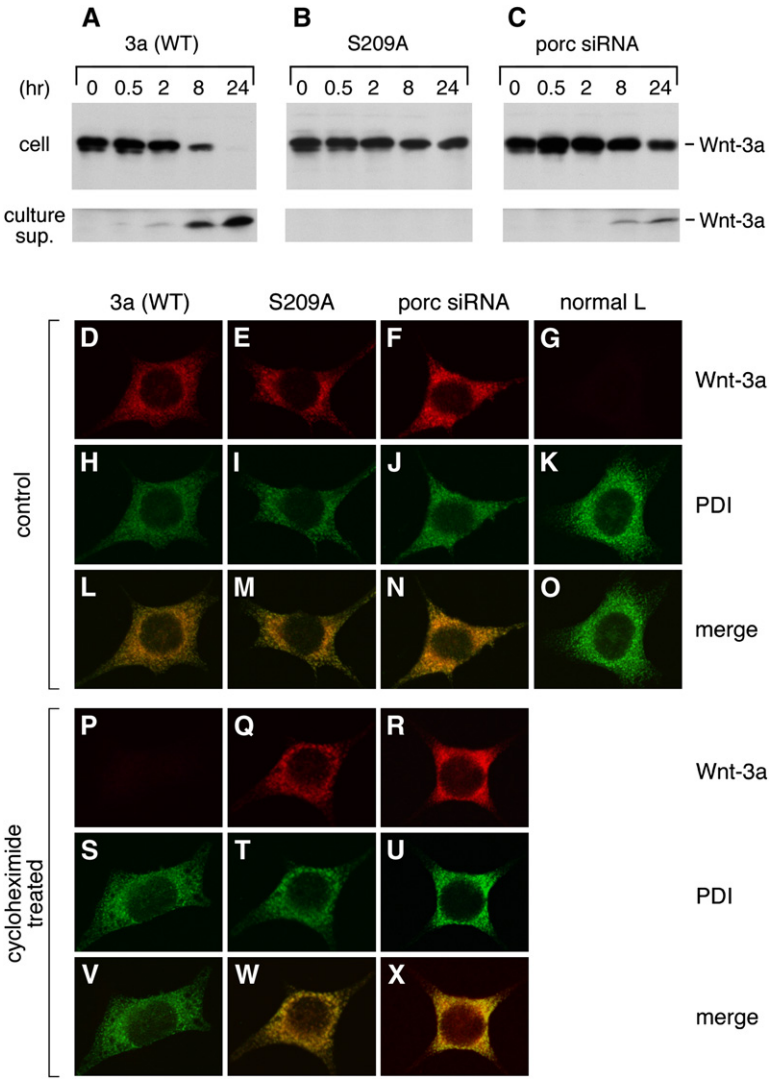

Figure 5. Defective Acylation Results in Preferential Retention of Wnt-3a Protein in the Endoplasmic Reticulum

(A–C) L cells expressing (A) wild-type Wnt-3a or (B) Wnt-3a (S209A-cl 1) as well as (C) wild-type Wnt-3a-expressing L cells defective in Porc activity (si21-6) were incubated with cycloheximide. The amounts of Wnt-3a remaining within cells ([A]–[C], upper panel) and released into the culture supernatant ([A]–[C], lower panel) were examined by immunoblotting at the indicated time points after the addition of cycloheximide.

(D–X) L cells expressing (D, H, L, P, S, and V) wild-type Wnt-3a or the (E, I, M, Q, T, and W) S209A mutant form, as well as (F, J, N, R, U, and X) wild-type Wnt-3a-expressing L cells defective in Porc activity and (G, K, and O) control L cells, were fixed 24 hr after incubation (D–O) without or (P–X) with cycloheximide. Cells were then stained with anti-Wnt-3a antibody (shown as red: [D]–[G] and [P]–[R]) or anti-PDI antibody, which is an ER marker (shown as green: [H]–[K] and [S]–[U]). (L–O and V–X) Merged pictures are also shown. The percentage of stained cells is shown in Table S2.

porc-deficient cells treated with cycloheximide (Figures 4C and 5C), although reduced levels of Wnt-3a secretion were detected, reflecting residual Porc activity. These results show that *porc* is required for secretion, as well as for Ser209-dependent acylation, of Wnt-3a from the cultured cells.

Wnt-3a with Defective Acylation Is Not Transferred from the ER

To determine the importance of acylation to Wnt-3a secretion, we examined the intracellular localization of Wnt protein with defective acylation (Figures 5D–5X; Table S2). Most Wnt-3a protein transcribed from the transfected gene is retained in the ER, probably due to limitations of protein trafficking from the ER (Figures 5D–5O). During cycloheximide-mediated inhibition of de novo protein synthesis, S209A protein was retained in the ER (Figures 5Q and 5W), whereas most newly synthesized wild-type Wnt-3a protein was secreted from cells under the same conditions (Figures 5P and 5V). Similarly, under the same conditions, most Wnt-3a protein was retained in the ER in *porc*-deficient cells (Figures 5R and 5X). Thus, Ser209-dependent acylation appears to be required for transit of Wnt-3a from the ER.

Ser209 Is Required for the Function and Appropriate Localization of Wnt-3a in *Xenopus* Embryos

To examine whether Ser209-dependent acylation would also be required for the function and secretion of Wnt-3a in vivo, we next injected S209A mRNA into *Xenopus* embryos (Figure 6). While the injection of wild-type Wnt-3a or S211A mRNAs into the ventral side of *Xenopus* eggs at the four (or two)-cell stage caused ectopic axis formation (Figures 6B and 6D; Table S3), as well as induction of the target genes *Siamois* and *Xnodal related-3* (Figure 6G), injection of S209A mRNA had no obvious effect, indicating that Ser209 is essential for the in vivo function of Wnt-3a (Figures 6C and 6G). Furthermore, whereas most wild-type Wnt-3a and S211A proteins were transported to the apical cell border, most S209A proteins were retained inside the expressing cells, probably in the ER (Figures 6I–6K). These results confirm that Ser209, which is required for acylation and secretion of Wnt-3a in cultured cells, is also essential for proper intracellular transport of Wnt-3a proteins in vivo.

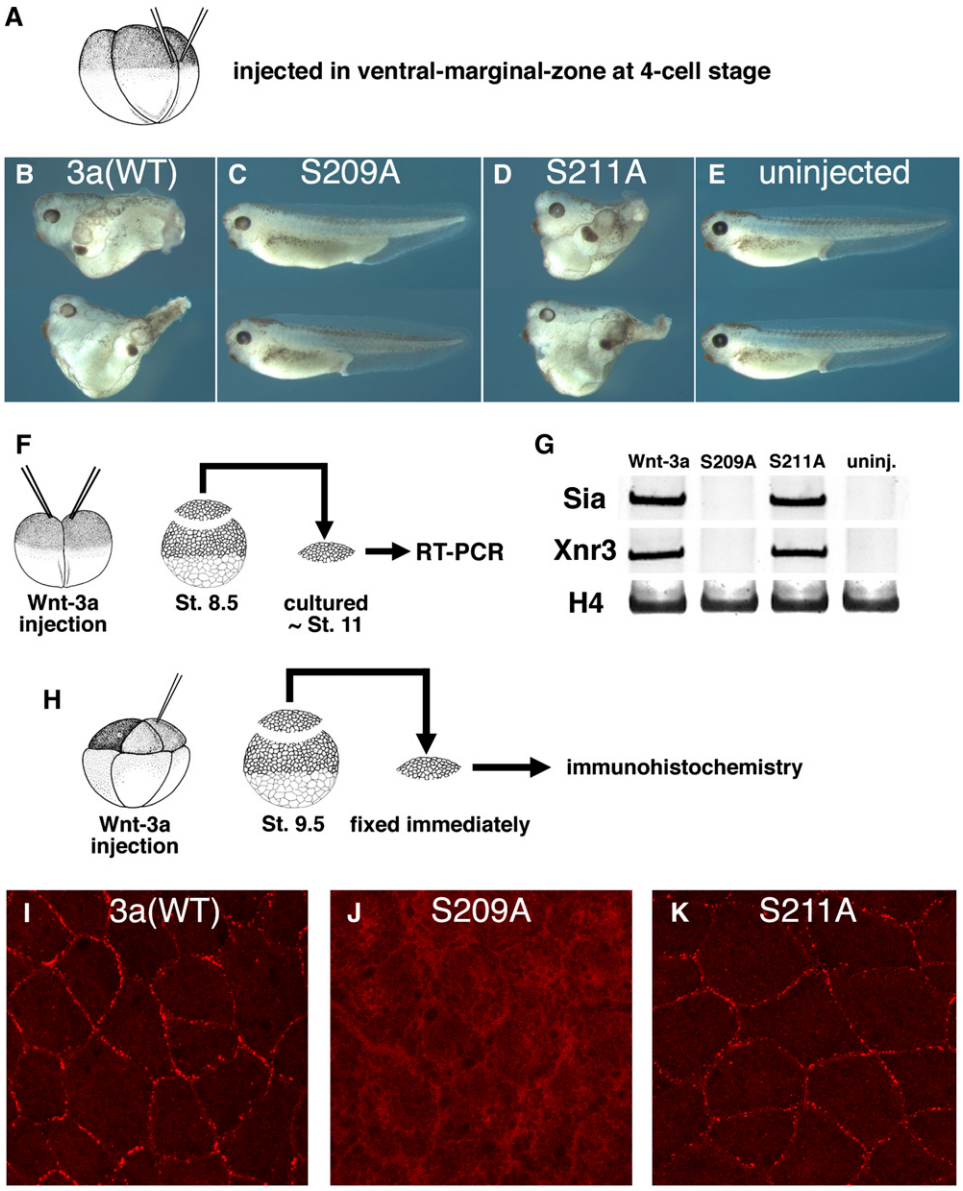

Figure 6. Acylation-Defective Wnt-3a Protein Is Largely Retained within the Cells of *Xenopus* Embryos

(A–K) (A), (F), and (H) indicate schematic representations of the experiments performed in (B)–(E), (G), and (I)–(K), respectively. (B–E) *Xenopus* embryos injected with mRNAs encoding (B) wild-type, (C) S209A, or (D) S211A forms of Wnt-3a, as well as (E) uninjected embryos, are shown. (G) Expression of *siamois* (Sia), *Xnodal related-3* (Xnr3), and *histone H4* (H4) in animal caps dissected from embryos injected with mRNAs encoding these forms of Wnt-3a, as well as in those from uninjected embryos, was analyzed by use of the reverse transcription-polymerase chain reaction. (I–K) Confocal images of animal caps dissected from the embryos injected with mRNAs encoding (I) wild-type, (J) S209A, or (K) S211A forms of Wnt-3a stained with anti-Wnt-3a antibody are shown.

Discussion

In this study, we demonstrated that Wnt-3a protein is acylated with an unsaturated fatty acid, palmitoleic acid (C16:1), at a conserved serine at the 209th residue, in addition to the previously reported Cys77 palmitoylation. We analyzed a number of mutant forms of Wnt-3a to demonstrate that Ser209 is required for appropriate trafficking of Wnt-3a protein from the ER during secretion, both in cultured cells and in embryos. In addition, Ser209-dependent acylation requires the function of Porc, a putative membrane-bound O-acyltransferase, in the ER. Thus, these results strongly suggest that Ser209-dependent acylation, catalyzed by Porc in the ER, is essential for transport of Wnt-3a from the ER during the secretion process.

Since acylation has been implicated in regulation of protein trafficking to intracellular organelles and particular domains of the plasma membrane (Smotrys and Linder, 2004; Huang and El-Husseini, 2005; Resh, 1999), Ser209-dependent acylation may be required for targeting of Wnt-3a proteins to specific organelles or membrane components required for secretion. Consistent with this model, an inhibitor of acyltransferase activity is known to inhibit the intracellular trafficking of Wg protein (Zhai et al., 2004). On the other hand, since

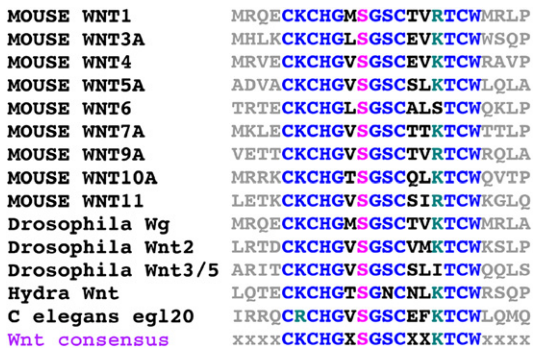

Figure 7. The Amino Acid Sequence Surrounding Ser209 Is Highly Conserved among Members of the Wnt Family

Aligned amino acid sequences surrounding Ser209 are shown for various forms of Wnt protein. Ser209 is shown in red, and other conserved residues are shown in blue. The consensus sequence among the members of the Wnt family is indicated at the bottom.

Wnt protein is transported between cells by lipoprotein particles in *Drosophila* wing imaginal discs (Greco et al., 2001; Panakova et al., 2005), an acylation-dependent trafficking system might be prerequisite for attachment of Wnt proteins to lipoprotein particles for secretion. Although it is not certain at present whether Wnt protein is secreted from cultured mammalian cells in association with lipoprotein particles, further characterization of the secreted Wnt protein might reveal whether acylation of Wnt protein has this proposed role in mammalian cell culture systems.

In addition, acylation may affect the function of several molecules involved in Wnt secretion, such as Wntless/Evi, a seven-pass membrane protein required for Wnt secretion in *Drosophila* and mammalian cultured cells (Banziger et al., 2006; Bartscherer et al., 2006), as well as the retromer complex, a protein complex involved in intracellular membrane trafficking and required for the long-range extracellular transport of Wnt protein in *C. elegans* (Coudreuse et al., 2006; Prasad and Clark, 2006). Because Wntless/Evi is primarily localized at the Golgi apparatus or plasma membrane, and since components of the retromer complex can be identified in *trans*-Golgi and trafficking vesicles, acylation-dependent trafficking of Wnt-3a from the ER appears to be prerequisite for Wntless/Evi and retromer complex function.

Another important aspect of this study pertains to the discovery of an anticipated lipid modification. This modification demonstrates several important characteristics, especially as related to the type of fatty acids involved. We demonstrated modification of Wnt-3a protein at Ser209 with monounsaturated fatty acids, such as palmitoleic acid (C16:1), but not with saturated fatty acids like palmitic acid (C16:0). Since the addition of deuterium-labeled palmitic acid (C16:0) into the cell culture medium resulted in a partial shift of the isotopic ion distribution of palmitoleic acid (C16:1) in mass spectrometry, Wnt-3a protein appeared to be modified with C16:1-fatty acid metabolically processed from palmitic acid (C16:0). Most cases of acylation with unsaturated fatty acids occur at sites where acylation with saturated fatty acids also occurs (DeMar et al., 1999; Dizhoor et al., 1992; Johnson et al., 1994; Kokame et al., 1992; Liang et al., 2001; Muszbek and Laposata, 1993; Neubert et al., 1992). In contrast, the results of our mass spectrometry analysis indicate a strong preference for modification with monounsaturated fatty acids at Ser209 of Wnt-3a. Given that cells store considerable amounts of saturated fatty acids as substrates for acyltransferases, the predominant modification with a palmitoleoyl (C16:1) moiety must be the result of preferential utilization of this unsaturated fatty acid as a substrate.

In addition to modification with a palmitoleoyl (C16:1) moiety, another interesting feature of Ser209 acylation involves the amino acid residue to which the acyl moiety is attached. In most cases of protein fatty acylation examined to date, including N-myristoylation, S-palmitoylation, and N-palmitoylation, a serine residue is not utilized for connecting to the acyl moieties, although there are a few exceptions, including attachment of oxyester-linked octanoate (C8:0) to serine in ghrelin, a growth-hormone-releasing peptide of 28 amino acids (Kojima et al., 1999; Smotrys and Linder, 2004). Here, we showed a different example of serine-linked O-acylation, where serine is used to bond a monounsaturated acyl moiety to a protein. Thus, Wnt-3a acylation demonstrates several unusual characteristics, suggesting that some specific machinery appears to be involved in this acylation.

One strong candidate for a player involved in this machinery is Porc. Since most Wnt acylation detected with the labeling assay of this study was dependent on Ser209 and was abolished in cells with markedly reduced Porc activity, *porc* appears to be required for acylation at Ser209. If this is the case, it will be important to elucidate the mechanism by which this enzyme utilizes unsaturated fatty acids and recognizes serine residues. Interestingly, additional members of the MBOAT family of membrane-bound O-acyltransferases, including acyl-CoA cholesterol acyltransferase (ACAT) and diacylglycerol acyltransferase (DGAT), utilize monounsaturated fatty acids as substrates (Cases et al., 1998; Seo et al., 2001). Thus, certain structural characteristics of this family may contribute to their preferential utilization of unsaturated fatty acids, as well as their role in O-acylation.

Another question pertains to the biological significance of acylation by unsaturated fatty acids. Although the functional significance of such acylation remains unclear, the bent structure produced by the double bond as a result of this process may influence the interaction of acylated proteins with lipid structures. Both in vivo and in vitro results indicate that acylation with unsaturated fatty acids results in displacement of proteins, including Fyn, annexinII, and Gai, from membrane domains within ordered lipid structures (Liang et al., 2001; Moffett et al., 2000; Zhao and Hardy, 2004). Thus, it seems unlikely that unsaturated fatty acylation enhances protein targeting into some ordered lipid structures, but rather that the folded structure might be advantageous for packaging fatty acid chains into the interior of small lipid particles. As such, we can speculate that palmitoleoyl (C16:1) modification may enable packaging of Wnt proteins into protein-lipid particles. An intracellular precursor of a lipoprotein particle might be a candidate for such a particle (Greco et al., 2001; Panakova et al., 2005).

Finally, it is of interest to note that the amino acid sequence surrounding Ser209 (C-K-C-H-G-(LIVMT)-S-G-S-C, where the bold S indicates Ser209) is highly conserved among members of the Wnt family, although the function of this motif remains unknown (Figure 7). Our results indicate that Ser209 in this conserved motif is essential for palmitoleoyl (C16:1) modification and secretion of Wnt-3a protein, suggesting that other Wnt members may likewise be modified by palmitoleoyl acid (C16:1), depending on the presence of this conserved motif. An attempt to reveal the role of this motif might provide clues regarding the molecular mechanism behind acylation.

Experimental Procedures

Cell Culture, Transfection, and Metabolic Labeling of Wnt Protein

L cells were cultured at 37°C in a 1:1 mixture of DMEM and Ham's F12 medium supplemented with 8.3% fetal calf serum and antibiotics. Stable transfectants expressing truncated or point-mutated forms of Wnt-3a were established as previously described (Shibamoto et al., 1998). Supernatants from cultures of Wnt-3a- or Wnt-5a-producing L cells were prepared as previously described (Shibamoto et al., 1998; Yamanaka et al., 2002). For inhibition of de novo protein synthesis, L cells were treated with 10 μg/ml cycloheximide for up to 24 hr; for that of Porc activity, a plasmid vector, pSilencer 3.0-H1 (Ambion), expressing siRNA specific for *porc* (si21: 5′-AAGTT GTCACAAGCTGGAACC-3′), was used to transfect Wnt-3a-expressing L cells. Stable transfectants with varying levels of defective *porc* expression were thus established and used in the experiments.

L cells secreting various forms of Wnt-3a (or Wnt-5a) and control L cells were inoculated into 35 mm dishes and were then incubated overnight at 37°C. Next, these cells were incubated for 36 hr in serum-free medium containing 0.37 MBq/ml [U-^{14}C] palmitic acid (30.4 GBq/mmol) or 14.8 MBq/ml [9,10-^{3}H] palmitic acid (2.2 TBq/mmol), or they were incubated in serum-free medium without labeled fatty acid for 20 hr and then with 0.37 MBq/ml [U-^{14}C] palmitic acid (30.4 GBq/mmol) for 4 hr. The culture supernatant or cell lysate was collected and immunoprecipitated with anti-Wnt-3a (or anti-Wnt-5a) antibody (R.T. and S.T., unpublished data) and protein G Sepharose beads. Immunoprecipitates mixed with sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 2-mercaptoethanol at the final concentration of 1.3 M were incubated at 37°C for 1 hr, separated by SDS-polyacrylamide gel electrophoresis, and visualized by autoradiography with a Kodak BioMax TranScreen LE system or by image analysis with a BAS 2500 (Fuji Photo Film).

Antibodies, Immunoblotting, and Immunocytology

The preparation of monoclonal anti-Wnt-3a and anti-Wnt-5a antibodies is described elsewhere (R.T and S.T., unpublished data). These antibodies specifically recognize Wnt-3a or Wnt-5a proteins, respectively. Anti-protein disulfide isomerase rabbit polyclonal antibody (Calbiochem), anti-HA antibody (Covance), and anti-FLAG M2 affinity gel (Sigma), were purchased. Immunoblotting was performed according to a standard protocol. For immunocytological analysis, cells were fixed with 3% paraformaldehyde in PBS(−) for 10 min at room temperature, made permeable by treatment with cold 100% methanol for 10 min, and then incubated with anti-Wnt-3a antibody and anti-protein disulfide isomerase (anti-PDI, a rabbit polyclonal antibody) for 1 hr at room temperature. Thereafter, the cells were probed with CY3-conjugated anti-mouse IgG and Alexa 488-conjugated anti-rabbit secondary antibodies and were observed under a confocal microscope (Carl-Zeiss, LSM510).

Nano-Flow Liquid Chromatography

FLAG-tagged Wnt-3a protein was purified with anti-FLAG M2 antibody from the culture supernatant of Wnt-3a-expressing L cells in serum-free medium. Purified Wnt-3a protein was reduced with 0.2 M dithiothreitol, and cysteine alkylation was carried out with monoacrylamide. Wnt-3a protein was directly applied to a gel for 8.0% SDS-PAGE, after which the gel was stained with silver-staining reagents or Coomassie brilliant blue (CBB). The protein band at ~45 kDa was excised from a strip of the gel. The excised protein band that had been stained with silver-staining reagents was destained with 20 mM EDTA 2Na-50 mM NH_4HCO_3 in 30% aqueous acetonitrile and was then washed with 15 mM potassium hexacyanoferrate(III)-50 mM sodium thiosulfate. The band stained with CBB was destained with 100 mM NH_4HCO_3 in 60% aqueous acetonitrile. The gel was then subjected to in-gel digestion with trypsin (Promega).

The trypsin digest was extracted from the gel with 0.1% trifluoroacetic acid in 60% aqueous acetonitrile. The resultant solution was injected into an Ultimate nano-LC system (Dionex), where the digested peptides were first concentrated with a C18 trapping column (0.3 mm × 1 mm, Dionex, Idstein, Germany) at a flow rate of 30 μl/min, and then separated by using a C_{18}-Pepmap column (0.075 × 150 mm, Dionex). A linear gradient of solvent A (0.1% trifluoroacetic acid in water) and solvent B (0.1% trifluoroacetic acid in acetonitrile) was used for the separation, and the peptides were eluted by increasing the concentration of solvent B from 5% to 80% over a period of 60 min at a flow rate of 200 nl/min. The effluent was monitored at 214 and 280 nm and was directly blotted at 1 min intervals onto the flat surface of a stainless steel plate (a MALDI sample plate) over a 96 min period. Thereafter, the matrix solution (5 mg/ml α-cyano-4-hydroxycinnamic acid) was blotted manually onto each sample spot and then dried.

Matrix-Assisted Laser Desorption/Ionization, MALDI, Mass Spectrometry

Overall peptide identification was carried out by using a MALDI-TOF/TOF (4700 proteomics analyzer, Applied Biosystems, Framingham, MA), followed by a database search with Mascot ver. 2.0 (Matrix Science, Manchester, UK). Ions were generated by irradiating the sample area with a 200 Hz Nd:YAG laser operated at 355 nm. Calibration was performed by using MH^+ ions from a mixture of angiotensin I (m/z 1296.6), dynorphin (m/z 1604.0), ACTH (1-24) (m/z 2932.6), and β-endorphin (m/z 3463.8). The isotopic envelopes of the observed ions were closely compared with the theoretical envelopes generated by *Isotopica*, a software aid for calculating and assessing complex isotopic envelopes (http://coco.protein.osaka-u.ac.jp/Isotopica) (Fernandez-de-Cossio et al., 2004). For MS/MS, the precursor ions were accelerated at 8 kV in MS1 and were fragmented in a collision cell by using air as the collision gas. The resultant fragment ions, re-accelerated at 15 kV, were analyzed in MS2 equipped with a reflectron. MS/MS spectra were interpreted by *SeqMS*, a software aid for de novo sequencing by MS/MS (http://www.protein.osaka-u.ac.jp/rcsfp/profiling) (Fernandez-de-Cossio et al., 2000).

Xenopus Injection, Detection of Target Gene Expression, and Immunohistology

For morphological analysis, in vitro-synthesized wild-type, S209A, or S211A Wnt-3a mRNA sequences were injected into the ventral marginal zones of *Xenopus* eggs at the four-cell stage (Sokol et al., 1991). To examine the expression of *Siamois* and *Xnr3* (Brannon and Kimelman, 1996; McKendry et al., 1997), we injected the various Wnt-3a mRNA sequences at the two-cell stage, after which the animal caps of the injected embryos were dissected at stage 8.5, cultured to stage 11, and analyzed for gene expression by use of the reverse transcription-polymerase chain reaction. The following specific primers were used: forward, 5′-GATAACTGGCATTCCTGAGC-3′; reverse, 5′-ACAAGTCAGTGTGGTGATTC-3′ (23 cycles) for *siamois*; forward, 5′-CCATGTGAGCACCGTTCC-3′; reverse, 5′-GAGCAAACT CTTAATGTAG-3′ (18 cycles) for *Xnr3*; and forward, 5′-ATAACATC CAGGGCATCACC-3′; reverse, 5′-ACATCCATAGCGGTGACGGT-3′ (18 cycles) for *histone H4* as the internal input control. For immunohistological analysis, the various Wnt-3a mRNA sequences were injected into the blastomeres in the animal side of eight-cell-stage blastulas, and the explants from the injected embryos were fixed at stage 9.5 in MEMFA (0.1 M MOPS [pH 7.4], containing 2 mM EGTA, 1 mM $MgSO_4$, and 3.7% formaldehyde) for 1 hr at room temperature. After bleaching in 10% H_2O_2 and blocking with FCS-PBS (PBS with 15% fetal calf serum) for 20 min, the explants were incubated overnight at 4°C with anti-Wnt-3a antibody in FCS-PBS. They were then incubated for 2 hr at room temperature with rhodamine-conjugated

rabbit anti-mouse IgG. The washed explants were mounted and observed under a confocal microscope (Carl-Zeiss, LSM510).

Supplemental Data

Supplemental Data include three figures and three tables and are available at http://www.developmentalcell.com/cgi/content/full/11/6/791/DC1/.

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