In vivo role of lipid adducts on Wingless

Xavier Franch-Marro1,*, Franz Wendler1,*, Janice Griffith2, Madelon M. Maurice2 and Jean-Paul Vincent1,‡

1National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA, UK
2Department of Cell Biology, University Medical Center Utrecht (UMCU), Rm G02.525, Heidelberglaan 100, 3584CX Utrecht, The Netherlands
*These authors contributed equally to this work
‡Author for correspondence (e-mail: jvincent@nimr.mrc.ac.uk)

Abstract

Two lipids (palmitate and palmitoleic acid) are appended onto Wnt proteins. It has been suggested that palmitate is required for signalling, whereas palmitoleic acid is necessary for progression through the secretory pathway. By mutating the relevant amino acids, we have investigated how these adducts contribute to the secretion and signalling activity of Wingless, the main Drosophila member of the Wnt family. Analysis of Wingless with a Cysteine 93 to Alanine mutation ([C77A]Wingless) shows that palmitoylation is essential for signalling activity in vivo (as well as in cultured cells). Moreover, without palmitate, Wingless fails to reach the surface of imaginal disc cells and, as electron microscopy (EM) analysis suggests, appears to accumulate in the endoplasmic reticulum (ER). Artificial targeting of palmitate-deficient Wingless to the plasma membrane does not rescue signalling activity. Therefore, palmitate at C93 has a dual role: in secretion and signalling. From our analysis of [S239A]Wingless, which lacks a conserved residue shown to be acylated in Wnt3a, we infer that palmitoleic acid is not, as previously suggested, absolutely required for secretion. Nevertheless, this mutant has poor signalling activity, suggesting that palmitoleic acid contributes significantly to signalling. We suggest that the overall level of lipidation affects signalling activity.

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Introduction

Lipid modification has recently emerged as an important feature of several signalling molecules. Lipid-modified secreted proteins include the members of the Hedgehog (Hh) and Wnt families, and, as shown recently, the Drosophila homologue of TGFβ, Spitz (Spi) (Miura and Treisman, 2006). Two distinct lipids are appended on Hh proteins: palmitate at the N-terminus and cholesterol at the C-terminus (Pepinsky et al., 1998); however, so far, Wnts and Spi are only known to be acylated (Willert et al., 2003; Miura et al., 2006; Takada et al., 2006). The hydrophobic nature of secreted lipoproteins raises interesting questions concerning progression through the secretory pathway, release from the surface of producing cells and transport to distant cells within a tissue. In this paper, we investigate how lipid modifications affect the secretion and signalling activity of Wingless, a Drosophila member of the Wnt family.

Two lipids have been shown to be appended onto Wnts: a palmitate on a cysteine and a palmitoleic acid on a serine. Palmitoylation at cysteine 93 (C93) of Wingless has been demonstrated by Willert et al. (Willert et al., 2003). The corresponding residue of mouse Wnt3a (C77) is also palmitoylated, suggesting that this modification is conserved. Indeed, replacement of this residue by alanine (C93A in Wingless) shows that palmitoylation is essential for signalling activity (in vivo as well as in cultured cells). Moreover, without palmitate, Wingless fails to reach the surface of imaginal disc cells and, as electron microscopy (EM) analysis suggests, appears to accumulate in the endoplasmic reticulum (ER). Artificial targeting of palmitate-deficient Wingless to the plasma membrane does not rescue signalling activity. Therefore, palmitate at C93 has a dual role: in secretion and signalling. From our analysis of [S239A]Wingless, which lacks a conserved residue shown to be acylated in Wnt3a, we infer that palmitoleic acid is not, as previously suggested, absolutely required for secretion. Nevertheless, this mutant has poor signalling activity, suggesting that palmitoleic acid contributes significantly to signalling. We suggest that the overall level of lipidation affects signalling activity.

In this study we use wing imaginal discs of Drosophila as an in vivo system in which to study the role of the acyl groups at C93 and S239 in Wingless. During the third-instar larval stage, Wingless is normally expressed in a row of cells that bisects the disc along the dorsoventral boundary. From there, it spreads throughout most of the presumptive wing region, where it binds to receptors of the Frizzled family (mainly Frizzled 2; Fz2) and activates downstream signalling. We find that, in imaginal discs, lipid modification at S239 is not absolutely required for secretion or membrane association but contributes to signalling activity. By contrast, mutation of C93 (no palmitoylation) causes inefficient exit from the endoplasmic reticulum (ER) and abrogates signalling. Our results suggest that dual lipidation is required for signalling activity. Lipidation also contributes to trafficking through the secretory pathway, although, in Drosophila Wingless, the role of palmitate predominates over that of palmitoleic acid, whereas the contrary is true for mammalian Wnt3a.
Results and Discussion

Acylation is required for signalling activity in vitro and in vivo

As reported by Galli et al. (Galli et al., 2007), Willert et al. (Willert et al., 2003) and Kurayoshi et al. (Kurayoshi et al., 2007), respectively, palmitoylation of Wnt1, Wnt3a and Wnt5a at an N-terminal cysteine is essential for signalling activity. We found that a mutation at the corresponding site in Wingless (C93A) abolishes signalling activity in cultured Drosophila S2 cells. S2 cells were co-transfected with a Fz2-expressing plasmid, a reporter plasmid (topflash) and a plasmid encoding either wild-type Wingless or [C93A]Wingless. A plasmid constitutively expressing Renilla luciferase was also included for normalisation and to provide a measure of cell health. Normally, three different forms of Wingless, representing different levels of glycosylation, can be distinguished by western blot (Tanaka et al., 2002). We found that [C93A]Wingless is also present in three forms (Fig. 1A), suggesting that it is expressed and modified normally. Nevertheless, C93A did not show any significant signalling activity (Fig. 1B). Using similar assays, we found that [S239A]Wingless is also modified normally by disc cells (Fig. 1A). Surprisingly, considering that [S209A]Wnt3a is inactive (Takada et al., 2006), [S239A]Wingless was found to have residual signalling activity (Fig. 1B).

In order to assess the in vivo significance of these in vitro observations, we generated transgenic flies expressing either Gal4-inducible HA-tagged [C93A]Wingless (UAS-HA-[C93A]Wingless) or [S239]Wingless (UAS-HA-[S239]Wingless). For control experiments, we used flies expressing HA-tagged wild-type Wingless (UAS-HA-Wingless). Strains expressing comparable amounts of protein, as assessed by western blot, were selected (data not shown). The transgenes were expressed under the control of the ap-Gal4 driver, which is active in the dorsal compartment of wing discs, including in one of the two rows of cells that normally express Wingless (see diagram in Fig. 1C). Using this driver, HA-Wingless causes 100% lethality at the pupal stage (not shown) (see Struhl and Basler, 1993). By contrast, expression of HA-[C93A]Wingless had no detectable effect: resulting flies were viable and no morphological defects could be seen even though mild activation of the Wingless pathway in the prospective wing blade causes the formation of ectopic bristles (Greaves et al., 1999). We conclude that [C93A]Wingless has no signalling activity in vivo. In the same assay, HA-[S239]Wingless prevented complete fusion of the notum but had no effect on wing patterning (e.g. no ectopic bristles). This is consistent with the weak activity seen in S2 cells. We conclude that palmitoylation at C93 is essential for signalling and that acylation at S239, although not essential, contributes significantly to signalling activity.

Distribution of [C93A]Wingless and [S239A]Wingless in imaginal discs

The distribution of wild-type Wingless, [S239A]Wingless or [C93A]Wingless was assessed by immunofluorescence following expression in imaginal discs with ap-Gal4. Using a conventional staining protocol that reveals both intra- and extra-cellular proteins, HA-tagged wild-type Wingless was detected in non-expressing cells.
in which it is known to be internalised (top half of Fig. 1D) (see also Piddini et al., 2005). By contrast, neither [S239A]Wingless nor [C93A]Wingless could be detected outside the expression domain (Fig. 1E,F). Therefore, reduction in lipiddation prevents Wingless from reaching target cells. Using a staining protocol designed to reveal only extracellular proteins, we confirmed that wild-type Wingless accumulates at the surface of expressing cells (Strigini and Cohen, 2000) (Fig. 1G,K). [S239A]Wingless also accumulated at the cell surface (Fig. 1H,L), hence, it must be able to progress through the secretory pathway. By contrast, no [C93A]Wingless could be detected at the surface of expressing imaginal disc cells (Fig. 1I,M). This suggests that secretion of this form of Wingless is impaired in vivo. Alternatively, [C93A]Wingless could be secreted but not retained at the cell surface. Overall, our in vivo results differ somewhat from those obtained in mammalian cell culture. In particular, in our assay, palmitate at C93 of Wingless appears to be more important for secretion than palmitoleic acid at S239.

Failure to reach, or accumulate at, the cell surface could explain why [C93A]Wingless is unable to signal. To test this possibility, palmitoylation-deficient Wingless was tethered at the cell surface with a heterologous transmembrane domain from the Neurotactin protein (NRT). We expressed NRT-HA-[C93A]Wingless in transgenic imaginal discs and compared its signalling activity to that of wild-type protein similarly tethered to the cell membrane; the latter is known to be highly active (Zecca et al., 1996). Both membrane-tethered forms of Wingless were expressed under the control of the ap-Gal4 driver and both were found to localise at the surface of expressing cells. In particular, Fig. 1J,N shows that NRT-HA[C93A]Wingless could be detected using the extracellular-staining protocol. Despite its localisation at the cell surface, NRT-HA[C93A]Wingless completely lacked signalling activity (adult flies did not show any phenotype and expression of senseless, a known target gene, was unaffected; not shown). This is in clear contrast to NRT-HA-Wingless, which is lethal at pupal stages because of the strong activation of target genes (Struhl and Basler, 1993). Therefore, restoring membrane localisation to [C93A]Wingless does not rescue signalling activity. We conclude that the palmitate moiety is strictly required for signalling activity, either for engagement with its receptor or to induce the appropriate conformational changes within the receptor upon binding.

[S239A]Wingless and [C93A]Wingless are secreted by S2 cells
The absence of detectable HA-[C93A]Wingless at the surface of imaginal discs suggests that palmitoylation could be essential for secretion in vivo. Alternatively, as suggested above, [C93A]Wingless could be secreted but would not be detectable for secretion in vivo. Alternatively, as suggested above, [C93A]Wingless could be secreted but not retained at the cell surface. Overall, our in vivo results differ somewhat from those obtained in mammalian cell culture. In particular, in our assay, palmitate at C93 of Wingless appears to be more important for secretion than palmitoleic acid at S239.

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outside the expression domain because of lack of retention at the cell surface and rapid dilution in the extracellular space. Willert et al. (Willert et al., 2003) favour the latter explanation because they found that palmitoylation-deficient Wn3a is secreted by mammalian cells in culture. To measure the secretion of Wingless variants by Drosophila S2 cells, we generated fusion proteins with firefly luciferase, measured the amount of luciferase activity in the medium and normalised it to the total amount of luciferase activity that was produced. We found that luciferase-[C93A]Wingless as well as luciferase-[S239]Wingless are secreted into the medium to the same extent as luciferase-Wingless (Fig. 2A). Therefore, we conclude that lipid modifications are not essential for secretion of Wingless from Drosophila cells in culture.

Palmitoylation of Wingless at C93 promotes binding to Frizzled 2

As shown above, [C93A]Wingless had no signalling activity, even when it was forced to accumulate at the cell surface with an exogenous transmembrane domain. This could be because [C93A]Wingless has a reduced affinity for Fz2. No direct quantitative assay is yet available to measure the binding of Wingless to full-length Fz2. As an alternative, we adapted a paracrine signalling assay to infer binding information. In this assay, cells expressing HA-Wingless or HA-[C93A]Wingless were co-cultured with cells carrying a reporter plasmid, a Fz2-expressing plasmid and a plasmid expressing Histone-GFP. The latter construct was included so that receiving cells could be distinguished from Wingless-producing cells by fluorescence microscopy. Measurements of reporter activity with luciferase assays confirmed that HA-[C93A]Wingless does not trigger signalling, whereas HA-Wingless does (Fig. 2B). Furthermore, we confirmed that wild-type Wingless accumulates within non-expressing receiving cells (Fig. 2C,E), presumptively following Fz2-mediated endocytosis (Piddini et al., 2005). By contrast, HA-[C93A]Wingless could not be detected within receiving cells (Fig. 2D,F). One likely interpretation is that HA-[C93A]Wingless does not bind Fz2 and hence cannot be internalised.

We next investigated whether an interaction between Fz2 and [C93A]Wingless could be detected when these proteins are expressed in the same cells. This is a less stringent assay because it does not require the ligand to travel in the extracellular medium in order to find its receptor on non-expressing cells. We found that co-overexpression of Fz2 and [C93A]Wingless leads to some accumulation of [C93A]Wingless at the cell surface in S2 cells (not shown). This is also the case in imaginal discs. As shown in Fig. 2G-J, overexpression of Flag-tagged Fz2 renders HA-tagged [C93A]Wingless detectable by extracellular staining. One possibility is that exogenous Fz2 could help [C93A]Wingless overcome a block in secretion, perhaps escorting it to the cell surface. Alternatively, [C93A]Wingless could independently reach the cell surface and be retained by Fz2. In either case, the above results suggest that Fz2 can interact (directly or indirectly) with [C93A]Wingless but to a much weaker extent than with wild-type Wingless. Such reduced affinity could account for the loss of signalling activity.

Retention of [C93A]Wingless in the ER of imaginal disc cells

The absence of [C93A]Wingless from the cell surface suggested an impairment in secretion. To further investigate this possibility, we turned to comparative immunofluorescence and immuno-EM. First, we set out to compare the localisation of wild-type Wingless and [C93A]Wingless by co-expressing these two forms, each with a distinct tag (HA for [C93A]Wingless and GFP for wild-type Wingless), and comparing the distribution by double immunofluorescence. For this, we used the epidermal cells of third-instar larvae because they are polyplid and hence large. As a control, HA-Wingless and GFP-Wingless (both wild type) were co-expressed. As expected, the subcellular localisation of the two tags (both reporting wild-type Wingless) was found to be indistinguishable (Fig. 3A-C). By contrast, expression of wild-type and [C93A]Wingless only partially overlapped in expressing epidermal cells (Fig. 3D-F). Note, in particular, the absence of [C93A]Wingless in large structures, possibly multivesicular bodies (MVBs), in which wild-type Wingless is present (arrowheads, Fig. 3D-F). In order to quantify this observation, we counted the number of vesicles that had GFP signal alone (wild-type Wingless), i.e. no HA signal (either [C93A]Wingless or wild-type for control). This was done in three different cells (three regions of interest each). We found threefold more single-labelled vesicles when HA-[C93A]Wingless and GFP-Wingless were co-expressed (34.6±7.9; n=9) than when HA-Wingless and GFP-Wingless were co-expressed (12.3±4.06; n=9). This was statistically significant (P=0.000118). Therefore, we conclude that the absence of the palmitate moiety affects Wingless trafficking, although we cannot, at the light-microscope resolution, distinguish between effects on endocytosis or secretion.

**Fig. 3.** Subcellular localization of wild-type Wingless and [C93A]Wingless in ectodermal cells of third-instar larvae (A-C) High-magnification view of a region of ectodermal cells co-expressing wild-type Wingless from two transgenes (one tagged with GFP and the other with HA) with the wg-Gal4 driver. Inset in A shows the whole cell. GFP is shown in green and HA in red. Extensive overlap is seen (yellow in A), including in large structures (arrowheads). (D-F) Co-expression of GFP-tagged wild-type Wingless (green) with HA-tagged [C93A]Wingless (red). Again, the whole cell is shown in the inset in D. Only partial overlap is seen between the mutant and wild-type proteins (D). Note, for example, the lack of colocalisation in large structures (arrowheads). Scale bars: 1 μm.
In order to characterise the trafficking differences between wild-type and [C93A]Wingless at the ultrastructural level, we turned to immuno-EM of imaginal disc cells. Strikingly, the overall number of gold particles in [C93A]Wingless-expressing cells was significantly higher than in cells expressing wild-type Wingless (Fig. 4A-D) even though the overall level of expression was the same for the two transgenes. Much of the [C93A]Wingless was in the ER (Fig. 4A,C). Indeed, quantitative analysis of the number of gold particles per unit of ER-membrane length revealed that [C93A]Wingless is present in the ER at a tenfold higher density than wild-type Wingless (compare overall staining in Fig. 4A,C and B,D). Wild-type Wingless was also enriched in the ER; however, it was consistently found at the plasma membrane, and particularly in MVBs (asterisk in Fig. 4G,H). In particular, despite showing frequent labelling for wild-type Wingless, MVBs were mostly devoid of [C93A]Wingless (asterisk in Fig. 4E,F). Because palmitate-deficient Wingless accumulates inside expressing cells, we conclude that, in vivo, the palmitate moiety is required for secretion. Most likely, the palmitate moiety is required for efficient transfer of Wingless from the ER to post-Golgi compartments.

Conclusion

Recent work with mammalian cell culture has suggested that the two lipid adducts appended to Wnt proteins might have specialised functions. Palmitate at C93 in Wnt1, at C77 in Wnt3a, C104 in Wnt5a or C93 in Wingless would be dedicated to signalling activity, whereas palmitoleic acid at S209 in Wnt3a or at S239 in Wingless would be essential for progression through the secretory pathway (Galli et al., 2007; Kurayoshi et al., 2007; Takada et al., 2006; Willert et al., 2003). With respect to secretion, we found that the role of lipids appears to be reversed in Drosophila Wingless, at least in vivo. We found that C93 is essential for progression through the secretory pathway, whereas S239 appears to be relatively dispensable in this function. We suggest, therefore, that the exact nature and position of the lipid adduct is not crucial for secretion and that different Wnts might use one or the other preferentially. Because [C93A]Wingless is secreted normally by S2 cells, it appears that the lipid requirement for secretion is somewhat relaxed in cultured cells, perhaps because they are not polarised. With respect to signalling, the overall level of lipiddation clearly matters, because both C93 and S239 are essential for in vivo activity. Considering that palmitate at C93 is required both for binding to Fz2 and for progression through the secretory pathway, it is conceivable that this lipid moiety could mediate binding to Wntless (also known as Evi and Sprinter), another seven-pass transmembrane, which contributes to Wingless secretion (Banziger et al., 2006; Bartscherer et al., 2006; Goodman 2006).

Fig. 4. [C93A]Wingless accumulates in the ER of wing disc cells and is absent from MVBs. (A,B) Portion of the apical surface of a disc cell. Empty space is the peripodial lumen (PL). HA-[C93A]Wingless and HA-Wingless (WT) expressed with dpp-Gal4 are visualized with anti-HA and a secondary antibody labelled with 15-nm gold particles. Arrowheads indicate examples of HA-positive ER areas. These are particularly abundant in the [C93A]Wingless sample. (C,D) Basolateral region of disc cells. Note again the relatively abundant labelling in the perinuclear and other ER areas of cells expressing [C93A]Wingless (C). See for comparison an equivalent area in wild-type-Wingless-producing cells (D). Arrowheads indicate examples of ER areas positive for Wingless labelling. (E,F) MVBs (*) in cells expressing high levels of [C93A]Wingless. Note the absence of label in these areas. (G,H) By contrast, anti-HA labelling is seen in the MVBs of cells expressing wild-type Wingless. G, Golgi unit; M, mitochondrion; N, nucleus; PL, peripodial lumen. ER is recognized by its characteristic morphology, as indicated from an independent preparation stained with anti-KDEL (see supplementary material, Fig. S1). Scale bars: 200 nm.
et al., 2006). Further biochemical work is needed to assess whether and how lipid modification affects the binding of Wnts to these receptors.

**Materials and Methods**

**Antibodies, plasmids and fly stocks**

Primary antibodies used were mouse anti-Wingless 4D4 (prepared from cells obtained from the DSHB), mouse M2 anti-Flag (Sigma; 1/1000), mouse anti-HA 1.1 (Covance; 1/500), rabbit anti-HA (Y-11) (Santa Cruz Biotechnology; 1/1000), rabbit anti-GFP (Abcam; 1/2500). Secondary antibodies (all from Molecular Probes) were Alexa-Fluor-488-conjugated goat anti-rabbit (1/200), Alexa488-conjugated goat anti-mouse (1/200), Alexa594-conjugated goat and anti-mouse (1/200) and Alexa-Fluor-594-conjugated goat and anti-rabbit (1/200). Expression plasmids for in vitro studies were derived from pMT/5-V5-HisA (Invitrogen), which allows induction of expression with copper ions. Wingless fusion genes were generated by inserting DNA encoding HA or HA–firefly-luciferase into the unique PstI site located near to the 5’ end of the wingless ORF. The C93A and S239A mutations were introduced using the QuikChange site-directed mutagenesis kit from Stratagene according to the manufacturer’s manual and verified by sequencing. The same protocol was used to introduce the C93A mutation in NRT-HA-Wingless (Struhl and Basler, 1993). For expression in transgenic flies, the various constructs were transferred into the pUAST vector. We used fly stocks carrying the following (Struhl and Basler, 1993). For expression in transgenic flies, the various constructs were transferred into the pUAST vector. We used fly stocks carrying the following.

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**References**


