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Porcupine-mediated lipidation is required for Wnt recognition by Wls

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ABSTRACT

Wnt proteins are members of a conserved family of secreted signaling ligands and play crucial roles during development and in tissue homeostasis. There is increasing evidence that aberrant Wnt production is an underlying cause of dysregulated Wnt signaling, however little is known about this process. One protein known to play a role in secretion is the transmembrane protein Wntless (Wls). However, the mechanism by which Wls promotes Wnt secretion is a riddle. It is not known which Wnt family members require Wls and what the structural requirements are that make some of them reliant on Wls for secretion. Here we present a systematic analysis of all known Drosophila Wnt family members with respect to their dependence on Wls function for secretion. We first show that the glycosylation status of Wg at conserved sites does not determine its dependence on Wls. Moreover, in apparent contrast to murine *wls*, Drosophila *wls* is not a target gene of canonical Wnt signaling. We then show that all Wnts, with the exception of WntD, require Wls for secretion. All Wnts, with the exception of WntD, also contain a conserved Serine residue (in Wg S239), which we show to be essential for their functional and physical interaction with Wls. Finally, all Wnts, with the exception of WntD, require the acyltransferase Porcupine for activity and for functionally interacting with Wls. Together, these findings indicate that Por-mediated lipidation of the S239-equivalent residue is essential for the interaction with, and secretion by, Wls.

Introduction

Wnt proteins are a family of cysteine-rich, secreted glycoproteins that are encoded in all animal genomes. Wnt signaling regulates multiple diverse processes during animal development and controls tissue homeostasis in the adult. Dysregulation of the pathway is the cause for several hereditary diseases, and is also associated with various cancers, including intestinal cancer (Clevers 2006; MacDonald et al., 2009). The signaling events triggered by Wnts are relatively well understood; however the mechanisms underlying Wnt secretion are only poorly mapped out. Given the emerging relevance of Wnt production as an underlying cause of dysregulated Wnt signaling closing this gap is imperative.

Briefly, what is known about Wnt secretion: newly synthesized Wnts are lipid modified in the ER — a reaction that reportedly requires the acyltransferase Porcupine (Por) (Kadowaki et al., 1996). From the ER, Wnts are transported to the Golgi complex, where they encounter the transmembrane protein Wntless (Wls) (Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). Wls supports transport of Wnts from the Trans-Golgi Network (TGN) to the plasma membrane. From the plasma membrane Wls (and maybe some Wnt protein) is retrieved by clathrin-mediated endocytosis; Wls is then routed by the retromer complex into a retrograde trafficking pathway back to the TGN (Belenkaya et al., 2008; Franch-Marro et al., 2008a, 2008b; Pan et al., 2008; Port et al., 2008; Yang et al., 2008).

The mechanism by which Wls promotes Wnt secretion is enigmatic. It is not known why Wnts require Wls for secretion, nor which Wnts depend on Wls or what the structural requirements are that make some of them Wls independent. To date, one Wnt has been reported to be Wls-independent: WntD (also known as Wnt8) (Ching et al., 2008). It plays a role in dorsal–ventral patterning in the Drosophila embryo as well as during the innate immune response in adult animals (Gordon et al., 2005). Interestingly, WntD was found to be free of lipid-modifications (Ching et al., 2008). This was somewhat surprising as lipid modification was generally presumed to be a universal feature of Wnt proteins. Is lipid modification a pre-determinant for the requirement of Wls?

To address these questions and to try to elucidate if there are other features which make Wnts dependent on Wls for efficient secretion we set out to systematically characterize the interaction of all seven Drosophila Wnts with Wls. Our findings suggest that the posttranslational modification of Wnts with lipid chains at a conserved Serine residue (Takada et al., 2006), corresponding to S239 in Wg, results in their recognition by Wls as cargo. Our results also point to Porcupine as being responsible for this modification in all the lipidated Drosophila Wnts. In contrast, neither the glycosylation status nor the type of signaling cascade activated by a Wnt is a determinant for the recognition by Wls.

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Material and methods

Fly stocks

Transgenics on landing sites ZH-22A for the *hsp70* promotor constructs or ZH-51D for the *UAS* promotor constructs:

Wg; Wg^{C93A}; Wg^{S239A}; $Wg^{C93A,S239A}$ ($Wg^{C,S}$); Wg^{N103A}; Wg^{N108A}; Wg^{N414A}; $Wg^{N103A,108A,414A}$ (Wg^{N3}); HAWg; $HAWg^{C93A}$; $HAWg^{S239A}$; $HAWg^{C93A,S239A}$; $HAWg^{N103A}$; $HAWg^{N108A}$; $HAWg^{N414A}$; $HAWg^{N103A,N108A,N414A}$; DWnt2; DWnt2-3xHA, $DWnt2^{S202A}$-3xHA; DWnt4; DWnt4-3xHA; $DWnt4^{S403A}$-3xHA; DWnt5; DWnt5-3xHA; $DWnt5^{S868A}$-3xHA ;DWnt6; DWnt6-3xHA; $DWnt6^{S283A}$-3xHA; DWntD; DWntD-3xHA; DWnt10; DWnt10-3xHA; $DWnt10^{S266A}$-3xHA; wls-lacZ; wls-wls(coding sequence); tubulinα1-porcupine-V5.

Other stocks:

yw ubiGFPnls hsp70-Flp tubGal80 FRT19; tubGal4 FRT19/FRT19
por^{2E} FRT19/FM7a
yw hsp70-Flp; Sp/CyO; wls^{1} FRT80/TM6B
yw hsp70-Flp ubiGFPnls FRT19; Sp/CyO
yw hsp70-Flp;; *dpp*-Gal4/TM6B
yw; UAS-Flp/CyO; *dpp*-Gal4/TM6B
yw hsp70-Flp;;FRT80 ubiGFPnls $M(3)i^{55}$
yw hsp70-Flp; UAS-HA-NRTwg/CyO

Clonings, construct generations

DWnt coding sequences were PCR amplified and cloned into pUASTattB, phsp70attB and pAc5.1 expression vectors containing a C-terminal 3xHA tag in frame using KpnI and BssHII restriction enzymes. Additionally we cloned the same coding regions without tag using KpnI/HindIII. The coding sequence of DWnt6 and DWnt10 had to be adjusted during the course of the study according to the recent annotation on Flybase and to fulfill criteria such as having a functional signal sequence (determined by using the SignalP 3.0 server (Bendtsen et al., 2004)). In the case of DWnt10 we had to replace the most N-terminal sequence by the Wg signal peptide because the predicted N-terminus did not encode for one (see alignment in Fig. S4).

The $WntD^{Wg235–246}$ construct was generated by exchanging 12 amino acids around S239 in Wg from Wg to WntD.

Protein accession numbers used for the phylogenetic tree:

Drosophila sequences were obtained from Flybase: Wg (FBpp0079060), Wnt2 (FBpp0087596), Wnt4 (FBpp0088345), Wnt5 (FBpp0074394), Wnt6 (adapted from FBpp0292343), WntD (FBpp0082243), and Wnt10 (adapted from FBpp0292305).
C. elegans sequences were obtained from Swiss-Prot: MOM2 (Q10459), Wnt1 (P34888), Wnt2 (P34889), LIN44 (Q27886), and EGL20 (A8WV58).
Zebrafish sequences were obtained from Swiss-Prot: Wnt7 (E7FBM9), Wnt11 (O73864), Wnt10A (P43446), Wnt5B (Q92050), Wnt2 (Q92048) or NCBI: Wnt16 (NP_001093516), Wnt7A (NP_001020711), Wnt4B (AF139536_1), Wnt2B (AAN62916), Wnt3 (NP_001108024), and Wnt5a (ABE96795).
Human sequences were obtained from Swiss-Prot: Wnt10B (O00744), Wnt4 (P56705), Wnt9B (O14905), Wnt3A (P56704), Wnt10A (Q9GZT5), Wnt7A (O00755), Wnt2B (Q93097), Wnt8B (Q93098), Wnt16 (Q9UBV4), Wnt5A (P41221), Wnt11 (O96014), Wnt9A (O14904), Wnt6 (Q9Y6F9), Wnt7B (P56706), Wnt2 (P09544), Wnt5B (Q9H1J7), Wnt3 (P56703), Wnt8A (Q9H1J5), and Wnt1 (P04628).

Secretion assay — in vivo

Hsp70-Wg/Wnt-3xHA transgenes on ZH-22A were combined with the wls^{1} FRT80 chromosome and balanced over the compound Cyo-TM6B balancer. After crossing the resulting stocks to a *yw* hsp70-Flp;; FRT80 ubiGFPnls $M(3)i^{55}$ strain, mitotic clones were induced by a 45 min. at 37 °C heat-shock 3 days AEL and the non-TM6B larvae were subjected to a 2nd heat-shock for 90′ at 37 °C 1 h before dissection to induce transgene expression.

Secretion assay — in vitro

S2R+ cells were transfected with the pAc5.1-Wg/Wnt-3xHA constructs using FuGeneHD (Promega). Equal numbers of transfected cells were then split and seeded in 96 well plates. The dsRNA for GFP as control and Wls were added to the serum free medium and cells were kept in SFM for 5 h before the media was supplemented with serum. After 3 days the supernatant (SN) was exchanged and replaced with fresh 200 µl Schneider's medium. After another 3 days the SN was collected and added to protein G-beads plus 0.2 µg of rabbit anti-HA (805 Santa Cruz) antibody for 2 h at 4 °C. The beads were washed twice in PBS the next day, the proteins were eluted from the beads in 20 µl sample buffer and reducing agent (Invitrogen) for 5′ at 95 °C and subjected to western blot analysis. The antibody heavy chain bands are shown as an immunoprecipitation loading control. Protein levels in the cell lysate were immunoblotted for α-HA and α-Tubulin as additional transfection controls.

Activity assay — in vivo

Each tagged and untagged UAS-Wnt construct was crossed into the *dpp*-Gal4 driver background. However, the constructs encoding Wg and or glycosylation mutant forms of Wg had toxic effects in the embryos and thus had to be established with a flp-out cassette between the UAS and the coding sequences; these constructs were crossed into a UAS-Flp;*dpp*Gal4 background. 3rd instar larvae were dissected and wing imaginal discs were stained for Senseless in combination with antibodies against Wg or the HA epitope.

Paracrine activity assays — in vitro

Kc cells were transfected with either the *wf*-Luc and *tubulinα1*-Renilla constructs or with the untagged pAc5.1-Wg/Wnt constructs using FuGeneHD (Promega). Cells were mixed the next day. Cells were lysed in passive lysis buffer (Promega) and the relative luciferase levels were measured on a Promega Glomax Multi Detection System after another 4 days. Cell lysate was immunoblotted for α-Wg and α-Tubulin as transfection controls.

Autocrine activity assay — in vitro

Kc cells were transfected with *wf*-Luc, *tubulinα1*-Renilla and the untagged pAc5.1-Wg constructs using FuGeneHD (Promega). Cells were lysed after 4 days in passive lysis buffer (Promega) and the relative luciferase levels were measured on a Promega Glomax Multi Detection System. Cell lysate was immunoblotted for α-Wg and α-Tubulin as transfection controls.

Immunoprecipitations (IP)

Co-IP's were performed on Kc cell lysate using ProteinG beads (Roche). Cell transfection was carried out using FuGeneHD transfection