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Fatty acid modification of Wnt1 and Wnt3a at serine is prerequisite for lipidation at cysteine and is essential for Wnt signalling

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ABSTRACT

The Wnt family of proteins is a group of extracellular signalling molecules that regulate cell-fate decisions in developing and adult tissues. It is presumed that all 19 mammalian Wnt family members contain two types of post-translational modification: the covalent attachment of fatty acids at two distinct positions, and the N-glycosylation of multiple asparagines. We examined how these modifications contribute to the secretion, extracellular movement and signalling activity of mouse Wnt1 and Wnt3a ligands. We revealed that O-linked acylation of serine is required for the subsequent S-palmitoylation of cysteine. As such, mutant proteins that lack the crucial serine residue are not lipidated. Interestingly, although double-acylation of Wnt1 was indispensable for signalling in mammalian cells, in *Xenopus* embryos the S-palmitoyl-deficient form retained the signalling activity. In the case of Wnt3a, the functional duality of the attached acyls was less prominent, since the ligand lacking S-linked palmitate was still capable of signalling in various cellular contexts. Finally, we show that the signalling competency of both Wnt1 and Wnt3a is related to their ability to associate with the extracellular matrix.

1. Introduction

The body of a multi-cellular organism is a highly organized structure of cells, tissues and organs. A handful of evolutionarily conserved cell signalling pathways is responsible for generating this structural complexity, both during development and its maintenance in adulthood. The Wnt pathway, initiated by secreted Wnt proteins, controls a remarkably diverse array of processes that include cell proliferation, differentiation, cell migration and cell polarity. Deregulation of Wnt signalling is implicated in a number of human disorders and cancer (reviewed in [1–4]). Currently, several different modes of the pathway have been recognized, with the majority of Wnt-dependent cascades requiring the seven-pass transmembrane Wnt receptor, Frizzled (Fz), the co-receptor low-density lipoprotein receptor-related protein (Lrp) and cytoplasmic protein, Dishevelled (Dvl). In canonical Wnt signalling the association of Wnt ligand with its corresponding receptors leads to the stabilization and accumulation of β-catenin protein via Dvl-dependent inhibition of the Axin, glycogen synthase kinase 3 (Gsk-3), and adenomatous polyposis coli (Apc) multi-protein complex. Subsequently, β-catenin enters the cell nucleus and together with the T-cell factor (Tcf)/Lymphoid Enhancer Factor (Lef) transcriptional regulators activates the expression of Wnt target genes [5] (a detailed summary on Wnt signalling can be found at the Wnt homepage http://www.stanford.edu/~rnusse/wntwindow.html).

The mammalian genome encodes 19 Wnt proteins of approximately 350–400 amino acids in length that contain an invariant pattern of 23–24 cysteines. It is presumed that many of these cysteine residues participate in the formation of intra-molecular disulphide bonds that stabilize proper folding of the polypeptide [6]. Although the degree of sequence identity between some Wnt family members is only 18%, it is thought that all Wnt proteins form a similar three-dimensional structure [7].

Mouse Wnt3a is post-translationally acylated by the attachment of two fatty acid adducts [8,9]. The modification by palmitic acid occurs at the first cysteine residue (C77) of the mature secreted protein, whilst the linkage of palmitoleic acid occurs at serine 209. In many Wnt ligands, the regions containing the acylated amino acids are homologous; thus it is thought that the majority of Wnt proteins are doubly-acyl-modified (reviewed in [10,11]). Indeed, S-acylation at the corresponding "prototype" C77 in Wnt3a was experimentally verified

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in chicken Wnt1 and Wnt3a, mouse Wnt5a, *Drosophila* Wingless (Wg; the Wnt1 orthologue) and Wnt8 [9,12–14]. The presence of the O-acyl moiety at positions homologous to S209 in Wnt3a has been studied in less detail; however, it was confirmed in Wg and chicken Wnt1 [12,15]. To date, *Drosophila* WntD is the only known non-lipidated member of the Wnt family [16]. Another common biochemical feature of Wnt ligands is N-linked glycosylation. The N-glycosylation status of approximately 11 different Wnts has been examined with all proteins displaying a distinct pattern of N-linked oligosaccharide attachment at multiple positions [6,17–20].

It has been well documented in both *Drosophila* and *C. elegans* that Wnts act as morphogens, initiating specific responses in relation to the amount of a particular Wnt protein in the extracellular space [21,22]. Thus, within a given tissue, the concentration of Wnt molecules can provide positional information to cells influencing their developmental fate. The effect of each post-translational modification on the Wnt "morphogenic" behaviour is rather elusive (reviewed in [23]). Interestingly, ectopically expressed mammalian Wnts are not easily diffusible and remain tightly associated with the cell surface [18,24,25]. It was suggested that the fatty acid moieties are responsible for this "stickiness" and limit Wnt long-range signalling [11]. However, the concentration gradient of Wg can be mediated by membranous exovehicles, so-called argosomes [26], or by lipoprotein particles [27]. These findings would rather support a positive role of lipid adducts in long-range signalling of Wg in *Drosophila* tissues.

In cultured mammalian cells, Wnt3a lacking the palmitic adduct is normally secreted, but its signalling activity is considerably perturbed [9,12,17]. This reduction is caused by a decreased affinity for the receptors, Frizzled or Lrp [17,28]. Similar results were obtained using corresponding mutants of Wnt5a and Wg [13,15]. However, the same Wg mutant was not functional in the *Drosophila* wing imaginal disc as it was retained in the endoplasmic reticulum [15]. Other discrepancies were also observed in studies involving O-acyl-linked and N-glycosylated modifications. For example, mutation of the acylated S209 residue of Wnt3a resulted in an inefficiently secreted protein, whereas the equivalent mutation (S239A) in Wg was released from *Drosophila* S2 cells with the same efficiency as wild-type ligand [8,15]. In Wnt1, the absence of N-linked oligosaccharide chains did not impair its activity and was properly secreted and induced transformation of Wnt-sensitive mouse mammary epithelial cells [19,25,29]. In contrast, N-glycosylation of Wnt3a and Wnt5a ligands was necessary for their efficient secretion. Finally, purified and subsequently enzymatically deglycosylated Wnt5a preserved its activity, but a non-glycosylated mutant form of Wnt3a was less active than its wild-type counterpart [13,17,30].

In the present study, we thoroughly examined the N-glycosylation and acylation status of mouse Wnt1 and Wnt3a ligands to determine how these post-translational modifications affect the secretion and signalling activities of these polypeptides. The activity tests included secondary axis formation in *Xenopus* embryos, reporter gene assays and real-time quantitative RT-PCR (qRT-PCR) analysis. Additionally, β-catenin stabilization, the hallmark of canonical Wnt signalling, was visualized by confocal microscopy in Wnt-producing cells. We demonstrated that fatty acid modification at the serine residue precedes and conditions subsequent palmitoylation of cysteine. Wnt ligands without any lipidic adducts were still N-glycosylated and secreted. In contrast, non-N-glycosylated Wnts displayed a decreased rate of secretion. This phenomenon could account for the lower activity of non-N-glycosylated ligands in paracrine signalling. Although we observed some discrepancies between the outcome of the experiments performed in *Xenopus* embryos and in cultured cells, both testing systems showed that the non-lipidated Wnts were completely inactive. In addition, our studies revealed functional and biochemical differences between Wnt1 and Wnt3a proteins. Particularly, the relationship between fatty acid content and activity was less stringent in Wnt3a since S-acyl-deficient ligand [Wnt3a(C77A)] – contrary to the corresponding Wnt1 variant [Wnt1(C93A)] – retained a substantial signalling activity. Moreover, Wnt3a(C77A) was deposited on the extracellular matrix (ECM) and released to culture medium with the same efficiency as the wild-type polypeptide. Interestingly, we never detected any Wnt1 in cell supernatants, which indicates that the majority of the extracellular protein binds to the cell surface or ECM. However, the ability of acyl-deficient Wnt1 mutants to adhere to ECM was severely impaired.

2. Materials and methods

2.1. Plasmids and lentiviral constructs

Constructs encoding mouse wild-type and mutant Wnt1 [31] and Wnt3a (kindly provided by O. Machon) proteins were generated in the mammalian lentiviral vector, pCDH1 (System Biosciences). Single or multiple amino acid substitutions were introduced into corresponding cDNA using a site-directed mutagenesis kit (Stratagene). Wnt1 and Wnt3a lacking the signal peptide (ΔNWnt1 and ΔNWnt3a, respectively) were generated by PCR and cloned into the pCDH1 vector [31]. PCR amplification steps were performed with Phusion High-Fidelity DNA Polymerase (Finnzymes). The truncated proteins were fused at the N-terminus to the Myc-tag (ΔNWnt1) or HA-tag (ΔNWnt3a). EGFP-tagged mouse Frizzled (Fz) 4, cloned into the pCS2 vector, was obtained from V. Bryja. The expression construct encoding Flag-tagged mouse Lrp5 was a kind gift from M. Semenov and X. He [32]. PCR-derived constructs were verified by sequencing; details of plasmid constructs are available on request.

2.2. Software and statistical analysis of data

Protein sequence alignments were performed using the MegAlign program (DNASTAR Lasergene 7). Signalling peptides and N-glycosylation sites were predicted using the Expert Protein Analysis System (ExPASy) at http://www.expasy.ch. Fisher's exact test was used to analyse the statistical significance of the results of the double axis formation assay. Data sets obtained in the gene reporter and qRT-PCR analyses were evaluated by Student's t-test.

2.3. Cell culture, transfections and generation of stable cell lines

Human HeLa, human embryonic kidney (HEK) 293, mouse L_{TK^-}, mouse 3T3 and mouse Wnt3a-producing L cells were purchased from ATCC. Rat2 and mouse Wnt1-transduced Rat2 fibroblasts (Rat2Wnt1) were kindly provided by A. Brown [33]. Rat2 cells producing wild-type mouse Wnt3a were described previously [34]. HEK 293 FT cells utilized for packaging lentiviral stocks were purchased from Invitrogen. Super-TOPFLASH HEK 293 (STF 293) cells containing the genome-integrated Wnt/β-catenin-responsive luciferase reporter, SuperTOPFLASH [35] were obtained from Q. Xu and J. Nathans. All cell lines were maintained in Dulbecco's modified Eagle's medium [(DMEM; purchased from Biochrom AG)] supplemented with 10% fetal bovine serum (Hyclone), penicillin, streptomycin and gentamicin (Invitrogen). Transient transfections were performed using Fugene HD (Roche). Lentiviruses were prepared using the Trans-Lentiviral Packaging System (Open Biosystems). Rat2 cells transduced with the corresponding recombinant lentiviruses were selected without subcloning using puromycin (Alexis; 5 μg/ml).

2.4. Antibodies, co-immunoprecipitations, western blotting and tunicamycin treatment

cDNAs encoding N-terminally His-tagged mouse Wnt1 [amino acids (aa) 225–370] and Wnt3a (aa 190–355) were subcloned into the pET28b vector (Novagen). Recombinant proteins were purified from bacterial [*E. coli*, strain BL-21 (DE3)] cell lysates by TALON affinity