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Isolation and characterization of recombinant murine Wnt3a

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Abstract

Wnt proteins are a family of morphogens that possess potent biological activity. Structure – function studies have been impeded by poor yield of biologically active recombinant Wnt as well as a propensity of isolated Wnt to self-associate in the absence of detergent. Using stably transfected *Drosophila* S2 cells, studies have been conducted to improve recovery of recombinant murine Wnt3a, establish conditions for a detergent-free Wnt preparation and examine the effects of limited proteolysis. S2 cell culture conditioned media was subjected to a 3-step protocol including dye-ligand chromatography, immobilized metal affinity chromatography and gel filtration chromatography. Through selective pooling of column fractions, homogeneous and purified Wnt3a preparations were obtained. Limited proteolysis of Wnt3a with thrombin resulted in site-specific cleavage within the N-terminal saposin-like motif. To generate detergent-free protein, Wnt3a was immobilized on Cu²⁺-charged, iminodiacetic acid-derivatized Sepharose beads, detergent-free buffer was applied and Wnt3a eluted from the beads with buffer containing imidazole plus 30 mM methyl- β -cyclodextrin (M β CD). Wnt3a recovered in M β CD-containing buffer was soluble and biologically active. Insofar as M β CD is a member of a family of non-toxic, low molecular weight compounds capable of binding and solubilizing small hydrophobic ligands, Wnt-cyclodextrin complexes may facilitate structure-activity studies in the absence of adverse detergent effects.

Keywords

murine Wnt3a; chromatography; methyl- β -cyclodextrin; *Drosophila* S2 cells; L cells; β -catenin; thrombin

Introduction

The “Wnt” proteins are morphogens that derive their name from the *Drosophila* *Wingless* gene and the mouse *INT-1* gene [1]. Mature murine Wnt3a is a secreted, 334 amino acid, glycosylated, cysteine-rich protein with a SDS-PAGE molecular weight of ~40 kDa [2]. In

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addition to Wnt3a, mammals possess another 18 Wnt genes that can be divided into 12 subfamilies on the basis of sequence similarity. Mammalian Wnt proteins share common features that are essential for their function, including a signal peptide, multiple glycosylation sites, numerous cysteine residues that form disulfide bonds [3] and a unique serine that serves as the site for covalent attachment of a long chain monounsaturated fatty acid [4]. Studies with Wnt proteins whose lipid attachment site has been mutated reveal that lipid modification is required for correct intracellular processing and biological activity [5].

Wnt signaling is largely conserved from *Drosophila* to humans and is known to regulate diverse embryonic processes including axis specification and organogenesis [6]. Furthermore, aberrant Wnt signaling has been implicated in a number of cancers, bone density phenotypes and neurological conditions [7]. Wnts exert their physiological effects through interaction with target cell surface receptors, termed frizzled and low-density lipoprotein receptor related protein (LRP) 5 or 6 (i.e. canonical Wnt signaling). Frizzled is a seven transmembrane spanning protein that serves as the primary receptor for Wnt while LRP5/6 are closely related single pass transmembrane proteins that function as Wnt co-receptors. Wnt3a binding to its cognate receptors induces stabilization of β -catenin by inhibiting a multi-protein degradation complex consisting of the adenomatous polyposis coli tumor suppressor protein, the scaffold protein, axin and glycogen synthase kinase 3. Upon stabilization, cytosolic β -catenin translocates to the nucleus where interaction with transcriptional regulators of the T cell factor/lymphoid enhancer factor family promote transcription of Wnt target genes.

Molecular studies of Wnt - cell surface receptor interactions requires recombinant Wnt protein. Although Wnt proteins elicit biological effects at very low concentrations, virtually all preparations include detergent. A recent X-ray crystal structure of *Xenopus* Wnt8 was achieved in the absence of detergent by co-expression with the cysteine-rich domain (CRD) of frizzled [8]. The structure revealed that frizzled CRD interacts with the fatty acyl moiety of *Xenopus* Wnt8, effectively sequestering it from the aqueous environment. Indeed, in the absence of detergent or frizzled CRD, this fatty acyl moiety likely contributes to the propensity of isolated Wnt proteins to aggregate. Building on the protocol originally developed by Willert et al [2] we now report an improved procedure for isolation of recombinant Wnt3a and introduce a novel strategy to eliminate detergent from isolated Wnt preparations.

Materials and Methods

Cell culture

Drosophila S2 cells stably transfected with murine Wnt3a (hygromycin B selection) were obtained from Dr. Roel Nusse (Stanford University) and cultured in Schneider's media (Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin and 500 mg/L L-glutamine (complete Schneider's media) at 22 °C. After 12–14 days of constitutive expression, the cells were used to expand the culture to 3 or 5 liters. Five hundred ml Bellco spinner flasks containing 300 ml complete Schneider's media were seeded with $0.5\text{--}1.0 \times 10^6$ cells/ml. The expanded culture was maintained, with stirring, for 14 days. Subsequently, the S2 cell

culture (the final concentration of cells was in the range of 4.5 to 5.5 million cells/ml) was centrifuged in 500 ml bottles at 8,000 rpm for 15 min at 4 °C. The cell pellet was discarded and the conditioned media (CM) collected and stored at 4 °C in 0.02 % sodium azide until processing. Prior to chromatographic separation of CM components, Tris was added to 20 mM, the pH was adjusted to 7.45 and the CM stored at 4 °C for an additional 2 to 3 days, during which a mineral precipitate comprised of media components, appeared. Triton X-100 was added to the decanted CM (1%; v/v) and the solution filtered through 0.45 µm membrane.

Dye-ligand Chromatography

Processed CM was applied to a 5 ml bed volume HiTrap Blue HP column (GE Healthcare). The column was protected from residual precipitate by an inline 0.45 µm syringe filter. The sample was applied to the column at ~2 ml/min with a peristaltic pump. The column eluate was collected during loading and α-Wnt3a immunoblot analysis performed to monitor Wnt3a binding to the column matrix. After loading up to 5 L processed CM and washing with 40 ml 20 mM Tris HCl, pH 7.45 containing 0.14 M NaCl and 1 % (w/v) CHAPS detergent (buffer A) the column was placed in an AKTA Prime FPLC system (GE Healthcare). All operations to this point were carried out at 4 °C. The column was washed with 150 ml buffer A at 2.5 ml/min and protein eluted with 1.5 M sodium chloride, 20 mM Tris-HCl, 1% CHAPS, pH 7.45 (buffer B) using the following gradient: 20 ml to 25% B; 5 ml to 35% B; 2.5 ml to 100% B followed by a further 75 ml at 100% B (flow rate = 2.5 ml/min). The collected fractions were pooled based on A₂₈₀ and SDS-PAGE analysis.

Immobilized metal affinity chromatography (IMAC)

Select Wnt3a containing fractions from the dye-ligand chromatography step (see Results) were pooled and applied to a 1 ml Cu²⁺ saturated HiTrap Chelating column at ~1 ml/min with a peristaltic pump. After loading the sample and washing with 5 ml buffer C (20 mM Tris HCl, pH 7.45, 0.5 M NaCl, 1% CHAPS), the column was placed in the AKTA Prime system and proteins eluted with an imidazole gradient using Buffer C and Buffer D (buffer C containing 1 M NaCl and 200 mM imidazole). The gradient was applied as follows: 0 % D to 2 % D (5 ml); 15 ml at 2% D; 2 % D to 16% D (5 ml); 16 % D to 40% D (5 ml) and 40 % D to 100% D (2 ml) followed by 20 ml at 100% D (flow rate = 1 ml/min). Fractions were pooled based on A₂₈₀ and SDS-PAGE analysis and concentrated to ~2 ml by centrifugal ultrafiltration (Amicon Ultracel 30 K; EMD Milipore, Billerica, MA).

Size exclusion chromatography

Pooled and concentrated Wnt3a-containing fractions obtained following IMAC were applied to the AKTA Prime system equipped with HP Sephacryl S-100 HR, 16 × 60 (GE Healthcare) equilibrated with 20 mM Tris-HCl, pH 7.45, 0.3 M NaCl, 1% CHAPS. Proteins were eluted with 70 ml buffer at flow rate of 0.5 ml/min (fraction size = 1 ml). Wnt3a containing fractions were combined and concentrated by centrifugal ultrafiltration.

Recombinant Wnt3a analysis

The recovery and purity of Wnt3a following each step of the purification process was assessed on the basis of total protein, protein stain following SDS-PAGE, quantitative immunoblot and cell-based activity assays (see below). α -Wnt3a immunoblot analysis was performed in triplicate and a four point calibration curve constructed using high purity Wnt3a whose concentration was determined by absorbance at 280 nm using a theoretical extinction coefficient = $67,390 \text{ M}^{-1} \text{ cm}^{-1}$. When necessary, absorbance values at 280 nm were corrected for light scattering using standard methods based on absorbance values at 320 nm and 350 nm [10]. SDS-PAGE was performed on 1 mm thick 10% acrylamide slab gels and stained with the Imperial Protein Stain (Pierce, Rockford, IL) or transferred to a PVDF membrane using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA). After blocking the membrane with 5% fat-free milk, α -Wnt3a (ab28472, Abcam, Cambridge, MA; 1:6,000 dilution) was added followed by HRP-conjugated goat anti-rabbit IgG (PI-1000, Vector Labs, Burlingame, CA; 1:10,000 dilution). Horseradish peroxidase-generated light was scanned on a FluorChem Q instrument (Alpha Innotech, San Leandro, CA). Images were quantified using ImageJ software, version 1.47v. Only gray images, containing a limited number of black pixels, were used for quantification. Isolated Wnt3a obtained from the gel filtration column was stable in CHAPS-containing buffer and could be stored without loss of activity at 4° C for a few weeks or, after flash-freezing in liquid nitrogen, at -80 °C for an extended period of time.

Luciferase reporter assay

Mouse L cells transfected with the Super TOPflash luciferase reporter variant [11; a gift from Dr. Roel Nusse] were employed. Assays were performed using the Luciferase Reporter Assay Kit (K-801-200, BioVision, Milpitas, CA). Luciferase activity was measured on a Wallac 1420 instrument (Perkin Elmer, Santa Clara, CA). Relative luciferase units were calculated from the initial slope of a plot of light intensity versus Wnt3a concentration.

β -catenin induction assay

L cells were cultured in high glucose DMEM containing 10% FBS and 100 U penicillin and streptomycin, overnight at 37 °C. Cells (0.5×10^6 per well, 12-well plate) were incubated with varying concentrations of purified Wnt3a for 3 h at 37 °C and harvested using RIPA cell lysis buffer (Santa Cruz Biotechnology, Dallas, TX) with protease inhibitor cocktail mix (Roche). Protein concentration of L cell lysates were measured and 10 μ g lysate protein applied to precast 10% polyacrylamide gels (Bio-Rad, Hercules, CA) and electrophoresed at 120 V. Separated proteins were transferred to a PVDF membrane and the membrane blocked as described above. Blots were probed with anti mouse β -catenin (BD Transduction Laboratories, San Jose, CA, 1:2,000 dilution) and anti-GAPDH (Millipore, Billerica, MA, 1:2,500 dilution) and positive bands detected with HRP-conjugated anti mouse secondary antibody (Vector Labs, Burlingame, CA, 1:5,000 dilution). Detected bands were analyzed as described above.

Limited proteolysis

Isolated Wnt3a was incubated in the presence and absence of thrombin (Sigma) at a 1:1 ratio for 4 h at 37 °C in 50 mM Tris, pH 7.5, 300 mM NaCl, 2.5 mM CaCl₂ and 1% CHAPS. Following incubation, SDS-PAGE was performed on 15% acrylamide slab gels that were either stained with Imperial Protein Stain or transferred to PVDF membrane for immune-detection or Edman degradation sequence analysis. α -Wnt3a immunoblot analysis was performed as described above and N-terminal sequencing was performed at the UC Davis Genome Center Molecular Structural Facility.

Detergent removal studies

Removal of CHAPS detergent and replacement with methyl- β -cyclodextrin (M β CD) was performed by binding 10 μ g purified Wnt3a (in CHAPS containing buffer) to 0.1 ml Cu²⁺-charged iminodiacetic acid (IDA) Sepharose beads (Sigma) in a 0.6 ml spin column (Pierce) for 1 h with gentle rotation. The beads were washed three times with 0.6 ml buffer E (40 mM Tris HCl, pH 7.45, 0.5 M NaCl and 30 mM M β CD). Subsequently, the Wnt3a-containing beads were incubated for 0.5 h in 0.25 ml buffer E containing 200 mM imidazole. A brief centrifugation at 100 x g was performed at each step. Two additional experiments were carried out in parallel, one with buffer E containing 1% CHAPS but no M β CD (positive control) and the other with buffer E with no CHAPS and no M β CD (negative control).

Results

Using *Drosophila* S2 cells stably transfected with murine Wnt3a, several attempts were made to enhance the yield and streamline processing of recombinant Wnt3a. Our approach was to introduce modifications to the method originally reported by Willert et al [2] and later modified [12]. Attempts to employ lectin affinity chromatography, cation exchange chromatography and use of lipoprotein deficient media in lieu of FBS, failed to significantly improve the yield or purity of the product. Ultimately, as described below, the best results were obtained using three chromatographic steps starting with 3 L S2 cell CM.

Dye-ligand chromatography

Whereas the protocol reported earlier [2] employed a 120 ml bed volume Blue Sepharose column, experiments revealed that the bed volume can be substantially decreased without adversely affecting Wnt3a binding or elution, while maintaining much lower sample volumes. To test Wnt3a binding capacity to this matrix, 5 liters processed S2 cell CM was applied to a 5 ml bed volume Hi Trap Blue Sepharose column. During loading, the flow-through was collected and monitored for Wnt3a by immunoblot analysis (Figure 1, **left inset**). The results showed that the first 2 L of column eluate, the second 2 L and the final 1 L, contained much less Wnt3a than the starting material (i.e. processed CM). The amount of Wnt3a detected in the eluate following application of the final liter of CM was more than the previous two 2 L samples, however, indicating that the binding capacity of the column for processed CM had been reached. Furthermore, small amounts of Wnt3a leached from the column during a subsequent 50 ml column wash (data not shown). During this wash, Triton X-100 was replaced by 20 mM Tris buffered saline (TBS) containing 1% CHAPS.

Detergent swapping served to change a low cost, strongly UV absorbing, high molecular weight micelle detergent for a high cost, non UV absorbing, low molecular weight micelle detergent. Subsequently, a two-step (shallow and steep) gradient of NaCl, from 150 mM to 1 M (70 ml total volume), followed by an extended high salt wash, was applied to the column with collection of 2.5 ml fractions (Figure 1). The elution profile, monitored for absorbance and conductivity, was comprised of a leading edge of absorbance, a main peak and a trailing edge. Immunoblot analysis (not shown) and general protein stain analysis (Figure 1 **right inset**) of column fractions revealed that Wnt3a was present in fractions across the entire profile. Although it contained immuno-detectable Wnt3a, the main protein peak (Pool 2; fractions 23–26) was discarded because it was heavily contaminated with other proteins. Thus, although unconventional, Wnt3a containing fractions on either side of the main protein peak were combined.

Immobilized metal affinity chromatography (IMAC)

The Wnt3a pool obtained following dye-ligand chromatography was applied directly to a 1 ml IMAC column charged with Cu^{2+} . Wnt3a bound to this column matrix and, after washing, was eluted with a three step 5 mM to 200 mM gradient of imidazole (Figure 2). The elution profile consisted of a main protein peak with a distinct trailing edge. Immunoblot analysis revealed that, once again, Wnt3a was present in all fractions, except for the sharp peak eluting at 10 ml. General protein stain analysis indicated that fractions at the beginning of the trailing edge possessed more contaminating proteins than later eluting fractions. As a result, two pools were made (Pool 1 and Pool 2). Pool 2 corresponded to the later eluting fractions that possessed less total Wnt3a but fewer contaminating proteins. Pool 1 and Pool 2 were each concentrated to 2 ml by centrifugal ultrafiltration.

Gel filtration chromatography

Sephacryl S100 gel filtration chromatography of concentrated IMAC Pool 2 yielded a protein profile comprised of a major peak and a minor peak (Figure 3). The major peak possessed small amounts of Wnt3a along with residual contaminating proteins. On the other hand, the later eluting peak possessed Wnt3a that was >85 % homogeneous. Note that the apparent “doublet” pattern above Wnt3a on the gel is not due to protein but, rather, can be attributed to the presence of β -mercaptoethanol [13].

Purification summary

A summary of the purification scheme for Wnt3a from S2 cell CM, including the selective pooling strategy employed, is shown in Figure 4. The relative purity of Wnt3a at various stages of the isolation procedure is shown in the middle panel. From this gel it is apparent that dye-ligand chromatography Pool 2 is heavily contaminated. Likewise, the relative purity of IMAC pools 1 and 2 can be seen. Finally, Wnt3a obtained following Sephacryl S100 gel filtration chromatography of IMAC Pools 1 and 2 is depicted. Whereas the Wnt3a obtained following gel filtration chromatography of IMAC Pool 2 is > 85 % homogenous, IMAC Pool 1 also yielded purified Wnt3a (see gel scan in lower panel), with residual contaminant proteins running between 20 to 25 kDa.

Activity measurements

The recovery and specific activity of Wnt3a during each stage of purification is presented in Table 1. The amount of total protein present in a given sample was estimated on the basis of UV absorbance at 280 nm while activity was measured using the Super TOPflash luciferase reporter assay wherein Wnt3a binding to frizzled/LRP5/6 on the cell surface leads to activation of the reporter gene. The results indicate that dye-ligand chromatography Pool 1 retained most of the Wnt3a activity present. Whereas IMAC Pools 1 and 2 had comparable levels of total Wnt3a activity units, the presence of contaminating proteins in Pool 1 lowered the specific activity in this pool. This result carried forward through the gel filtration step wherein chromatography of IMAC Pool 1 yielded a similar total activity, but lower specific activity. The best results were obtained when IMAC Pool 2 was subjected to gel filtration chromatography. In this case, the specific activity of the final product was much higher.

Effect of Wnt3a on β -catenin levels in L cells

The Super TOPflash luciferase reporter assay is highly sensitive and amenable to simultaneous activity measurements in a 96 well format. At the same time, it requires dedicated equipment, expensive reagents and is time consuming. Thus, we sought an alternative, low cost convenient assay method for routine evaluation of the effect of specific perturbations (limited proteolysis, detergent removal, chemical modification etc.) to recombinant Wnt3a. One of the most direct and quantitatively significant changes induced by Wnt interaction with its cognate receptors is production/stabilization of β -catenin [7]. However, in most cells β -catenin also functions as a cytoskeletal protein such that any increase in β -catenin levels induced by exposure to Wnt3a will be incremental, occurring against a background of cytoskeletal β -catenin. Whereas Mikels and Nusse [11] introduced a centrifugation step to separate cytosol from cytoskeleton in HEK 293 cell lysates, we took advantage of the finding that, in the absence of Wnt stimulation, mouse L cells do not contain detectable levels of β -catenin [14]. Exposure of these cells to increasing amounts of isolated Wnt3a induced a concentration-dependent increase in β -catenin (Figure 5). Incubations with 20 ng Wnt3a for 3 h led to the appearance of a β -catenin signal in whole cell lysates. Longer cell incubation times (e.g. 24 h) with Wnt3a increased the intensity of the β -catenin signal (data not shown). Considering a 3 h Wnt3a sample incubation with L cells, activity readouts can be obtained after ~8 h, with relatively few manipulations.

Limited proteolysis

The X-ray structure of *Xenopus* Wnt8 reveals a novel protein fold comprised of two distinct domains [8]. Whereas the N-terminal domain possesses a palmitoleoyl moiety covalently attached to a conserved serine residue within a saposin-like sequence motif, the C-terminal domain is smaller and adopts a molecular architecture similar to a cytokine fold [15]. Computer assisted analysis of the murine Wnt3a sequence for potential protease cleavage sites predicted a single thrombin cleavage site located near the C-terminus. By comparison with the *Xenopus* Wnt8 X-ray structure, cleavage at this site was predicted to yield two fragments that, under nonreducing conditions, should remain attached via an inter-chain disulfide bond. Upon incubation of isolated Wnt3a with thrombin at low thrombin:Wnt3a ratios, no cleavage was detected after 24 h. When the ratio was changed to 1:1, however,

quantitative cleavage occurred within 4 h, giving rise to two distinct lower molecular weight fragments (Figure 6). When SDS-PAGE was performed under non-reducing conditions, two fragments were still present indicating they are not linked via a disulfide bond. This observation, together with the apparent electrophoretic mobility of the fragments (~26 kDa and ~21 kDa), suggested cleavage had not occurred at the predicted site, but, rather, at an alternate site. α -Wnt3a immunoblot analysis (using an antibody specific for C-terminal residues 250–350) revealed the band of higher apparent molecular weight corresponds to a C-terminal fragment while the 21 kDa lower molecular weight band was not recognized by this antibody. In order to determine the site of thrombin cleavage, Edman degradation analysis was performed on both fragments. The results obtained reveal the N-terminal sequence of the ~21 kDa thrombin cleavage fragment corresponds to the N-terminus of mature murine Wnt3a (Ser-Tyr-Pro-Ile). On the other hand, ~26 kDa thrombin cleavage product gave rise to an N-terminal sequence corresponding to mature Wnt3a residues 163–167 (Ser-Ala-Met-Asn). Residue 162 of mature murine Wnt3a (the P' site) corresponds to Arg, in keeping with the known cleavage specificity of thrombin.

CHAPS replacement studies

A persistent problem observed with isolated Wnt preparations relates to its propensity to aggregate in the absence of detergent. Whereas Janda et al [8] circumvented this by complexing *Xenopus* Wnt8 to frizzled CRD, this approach is not suitable for *in vivo* or cell-based activity assays. Analysis of the X-ray crystal structure of *Xenopus* Wnt8 together with the relative abundance of polar and charged amino acids throughout the Wnt3a sequence suggests the covalently bound fatty acid moiety adversely affects Wnt's solution behavior. To test whether a reagent capable of sequestering this highly exposed hydrophobic fatty acyl chain could substitute for detergent, studies were conducted with M β CD. Purified Wnt3a (in CHAPS containing buffer) was bound to three separate 0.1 ml samples of Cu²⁺-charged IDA Sepharose beads present in centrifugal columns. The columns were washed 3 times with 0.6 ml TBS containing a) 1% CHAPS; b) 30 mM M β CD or c) TBS alone, respectively. Following equilibration of the beads with the corresponding buffer condition, bound Wnt3a was eluted with each buffer containing 200 mM imidazole. The results obtained (Table 2) indicate that, whereas CHAPS was most efficient at retaining Wnt3a solubility and activity, M β CD showed a positive effect compared to TBS alone, especially in terms of activity. Of the Wnt3a recovered following elution from the IDA Sepharose beads, 27 $\pm$ 2% of the M β CD-containing sample was recovered in the supernatant fraction following centrifugation versus 13 $\pm$ 2% for Wnt3a in TBS alone. Based on the fact that aggregated Wnt3a can be pelleted by centrifugation, we determined that centrifugation at 10,000 $\times$ g for 4 min effectively removes aggregated Wnt3a from the supernatant (data not shown). When this assay was applied to the Wnt3a samples, relative to Wnt3a in CHAPS-TBS, 73 $\pm$ 2% Wnt3a in the M β CD-containing TBS was recovered in the supernatant while 11 $\pm$ 19% of the Wnt3a in TBS alone was present in the supernatant. This finding indicates M β CD is protective against Wnt3a aggregation.

Discussion

Studies of Wnt structure - function relations have been hampered by difficulties obtaining significant quantities of protein. Whereas isolation of biologically active recombinant Wnt has been achieved [2], in general, the yield is low and the isolated protein requires detergent to prevent aggregation/loss of activity. Studies reported herein sought to optimize and streamline the expression and purification scheme in an effort to generate sufficient quantities of isolated Wnt for structure-function studies. The protocol employed resulted in high specific activity Wnt3a from stably transfected S2 cell CM in three steps, including dye-ligand chromatography, metal affinity chromatography and gel filtration chromatography. All chromatography steps were conducted in the presence of CHAPS detergent, which was required to maintain solubility and prevent aggregation. Whereas the protocol employed produces highly purified Wnt3a, the overall yield remains low, ranging from 6–13 µg purified Wnt3a per liter of S2 cell CM. There appears to be room for improved recovery of isolated Wnt3a from S2 cell cultures, however, since the starting CM had estimated Wnt3a levels of up to 350 µg/L. Using an alternative approach, Janda et al. [8] successfully co-expressed *Xenopus* Wnt8 in complex with the CRD of the Wnt receptor, frizzled 8, and employed the Wnt8-CRD complex for X-ray crystal structure determination. This tour de force achievement revealed that the palmitoleic acid moiety covalently bound to Ser187 of *Xenopus* Wnt8 is sequestered from the aqueous milieu by deep penetration into a binding pocket on frizzled CRD. Indeed, the palmitoleoyl chain embeds in the CRD, functioning as the key interaction site between Wnt8 and frizzled CRD. A consequence of this sequestration is protection of the acyl chain from solvent exposure-induced Wnt aggregation. On the basis of amino acid sequence composition alone, Wnt8 and Wnt3a are not hydrophobic proteins. Indeed, *Drosophila* WntD, which is not lipidated or glycosylated, can be expressed at relatively high levels with a lesser requirement for detergent [16, 17]. In the case of *Xenopus* Wnt8, sequestration of the otherwise exposed acyl chain by complex formation with frizzled CRD was key to their crystallization strategy. Another important finding of this study is that lipid modified Wnts are fatty acylated at a single site [4].

Whereas it may be anticipated that a non-lipidated Wnt would behave in solution as a soluble monomer, such variants lack biological activity [5]. This finding suggests covalent fatty acid attachment is essential for one or both of Wnt secretion/biological function. Based on this, it is necessary to utilize an expression system for recombinant Wnt that generates a lipidated protein product. Whereas this problem was solved by co-expression of Wnt8 with frizzled CRD [8], this approach is not optimal for biochemical analysis of receptor-ligand activity studies since the CRD is anticipated to compete with natural receptors that mediate Wnt signaling activity.

Wnt solubilized in CHAPS detergent micelles is the predominant form in which *in vitro* studies have been conducted to date and commercial sources of CHAPS solubilized Wnt exist, albeit at high cost. CHAPS properties include a small micelle size (6,600 MW), low aggregation number (i.e. 10) and a critical micelle concentration of 8 to 10 mM. At the same time, CHAPS is toxic to cultured cells [18]. In the case of long-term studies, for example with stem cells, CHAPS toxicity can be a problem, especially if repeated additions of Wnt are necessary to maintain a given culture. For protein biochemistry studies, given the

zwitterionic nature of CHAPS, it is presently unclear if Wnt – CHAPS mixed micelles simply sequester the palmitoleoyl moiety or if detergent molecules interact with/distort other regions of the protein.

An approach to circumvent detergent-related problems was developed by Morrell et al. [19]. These authors tethered Wnt3a to the surface of liposomes. In this system, Wnt3a predominantly associated with the exofacial surface of the liposome bilayer. Studies showed that Wnt3a-containing liposomes displayed enhanced and sustained signaling activity *in vitro* and characterization studies revealed that liposome-associated Wnt3a adopts a conformation that mimics native Wnt *in vivo*. Consistent with this, following subcutaneous administration, Wnt3a-containing liposomes were shown to induce hair follicle neogenesis, an established measure of regenerative capacity. Furthermore, Wnt3a liposomes have been successfully used in *in vivo* bone regeneration studies [20, 21]. Detracting from routine application of liposomal Wnt, the complexes are somewhat bulky and it is difficult to control the orientation of bound Wnt unless it is added to preformed liposomes. This, however, is a challenge since available Wnt preparations employ CHAPS, an agent that disrupts liposome structure.

In 2012, Mulligan et al [22] reported on a lipocalin-like protein, termed “secreted wingless interacting molecule”, and its ability to bind Wingless, the *Drosophila* homolog of Wnt. Lipocalins are known to bind hydrophobic ligands and, based on the X-ray structure of *Xenopus* Wnt8, it may be inferred that this protein binds and sequesters the palmitoleoyl moiety of Wingless, conferring water solubility to the protein in a manner that allows it to diffuse as a morphogen. In addition to lipocalin-like binding proteins, it has been reported that Wingless is transported between cells in association with the hemolymph lipoprotein, lipophorin [23]. Lipophorin exists as a roughly spherical noncovalent assembly of lipid and protein and, based on this structure, it is likely that interaction between Wnt and lipophorin is mediated by insertion of its fatty acid moiety into the surface monolayer of the lipoprotein. In this manner Wingless may become stably associated with the particle, enhancing protein solubility and facilitating its transport/presentation to distant cells.

In studies of Wnt mediated effects on myogenic cells, Portilho et al [24] attempted to deplete cholesterol from membrane raft domains using M β CD. These authors found that exposure of cells to M β CD significantly increased the amount of soluble Wnt3a in conditioned medium and concluded that cholesterol depletion alters the cell membrane association properties of Wnt3a, thereby increasing its concentration in conditioned medium. While this interpretation is plausible, an alternative hypothesis is that M β CD interacts directly with Wnt, promoting its solubility. In considering the X-Ray structure of Wnt8 and the manner in which the palmitoleoyl moiety is exposed at one end of the protein, we hypothesized that M β CD provides a cage for the fatty acyl chain, leading to enhanced solubility of monomeric, biologically active Wnt. Cyclodextrins are generally comprised of 6–8 glucopyranoside units, organized as a toroid. Whereas the interior of the toroid is not hydrophobic, the fact that it is less hydrophilic than the surrounding aqueous environment allows it to “host” hydrophobic molecules. On the other hand, the exterior of the toroid is hydrophilic, imparting water solubility to cyclodextrins. The report by Saito et al [25] that M β CD interacts with prenylated proteins led us to investigate whether Wnt3a may be

stabilized by interaction with M β CD in the absence of detergent. Whereas significant improvement was observed compared to buffer alone in terms of Wnt3a solubility and activity, the overall yield was suboptimal. It is possible that other cyclodextrins have a preferred “fit” with the palmitoleoyl moiety of Wnt3a such that studies with alternate forms of cyclodextrin may improve the interaction.

With respect to Wnt3a biological activity, a key event in canonical Wnt signaling is stabilization of a pool of β -catenin that is then able to migrate to the nucleus and serve as a transcriptional activator. On the other hand, it is recognized that β -catenin is a dual function protein, also participating in cell–cell adhesion complexes as part of a protein complex that forms adherens junctions. These cell–cell adhesion complexes are necessary for creation and maintenance of epithelial cell layers and barriers, forming a dynamic link to the actin cytoskeleton. Herein, we took advantage of the fact that mouse L cells do not employ β -catenin as a cytoskeletal protein [14] and hence, following Wnt3a stimulation, β -catenin signal intensity directly reflects Wnt signaling activity. This provides a relatively simple means to measure Wnt biological activity via immunoblot analysis of β -catenin levels in whole cell lysates.

One of the goals of this study was to employ this assay to examine the effect of limited proteolysis on Wnt3a biological activity. We focused on thrombin-mediated proteolysis since sequence analysis predicted a single thrombin cleavage site in Wnt3a. However, contrary to the predicted cleavage at Arg-Gly in the C-terminus of Wnt3a, a specific cleavage occurred at an Arg-Ser site in the N-terminal saposin-like domain. Given that thrombin is known to recognize Arg-Ser as a viable cleavage site [26], it appears that the C terminal Arg-Gly site may not be accessible to the enzyme, perhaps owing to interference from CHAPS micelles. It will be of interest to explore the Wnt3a saposin-like domain further for thrombin sensitivity as a function of lipid interaction since many saposins function via membrane interaction [27]. The fact that the fatty acylation site on Wnt is part of an extension of the saposin like domain, suggests that this motif may play a role in modulating Wnt membrane interaction and, possibly, exposure of the fatty acyl chain to its binding site on frizzled.

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Highlights

- Murine Wnt3a was isolated in 3 steps from stably transfected *Drosophila* S2 cell conditioned medium
- Wnt3a produced in this system activated β -catenin dependent signaling in L cells.
- Limited thrombin digestion resulted in site-specific cleavage in the Wnt3a saposin-like motif.
- Detergent replacement by methyl β cyclodextrin retains Wnt3a solubility and biological activity

Figure 1. Dye-ligand chromatography of S2 cell conditioned media

Five L of processed CM was applied to a 5 ml bed volume HiTrap HP column, unbound proteins were collected and 10 μ l aliquots analyzed by α -Wnt3a immunoblot (left inset). Lane A) processed CM; Lane B) first 2 L of column eluate; Lane C) second 2 L of column eluate and D) fifth liter of unbound eluate. Bound proteins were subsequently eluted by application of a NaCl gradient and collection of 2.5 ml fractions. Absorbance at 280 nm (continuous line) and conductivity (dotted line) are shown on the graph. Right inset depicts SDS-PAGE analysis of individual column fractions (indicated by numbers). The migration position of Wnt3a is depicted by the arrow. Molecular weight markers are shown at left.

Figure 2. IMAC of dye-ligand chromatography pool 1

Select fractions from the dye-ligand column elution profile were pooled and applied directly to a 1 ml IMAC column. After loading, bound proteins were eluted with a three step gradient of NaCl (0.5 M to 1.0 M) and imidazole (5 mM to 200 mM) with collection of 1 ml fractions. Absorbance at 280 nm (continuous line) and conductivity (dotted line) in fractions are shown on the graph. Inset) SDS-PAGE analysis of IMAC column fractions. The band corresponding to Wnt3a is depicted by the arrow at left. Molecular weight markers are shown at left.

Figure 3. Gel filtration profile of IMAC Pool 2

Fractions pooled from the IMAC chromatography step were concentrated to 2 ml and applied to a Sephacryl S100 column. Proteins were eluted in 1 and 3 ml fractions with monitoring of absorbance at 280 nm. Inset) SDS-PAGE analysis of column fractions. The band corresponding to Wnt3a is depicted by the arrow at left. Molecular weight markers are shown at left.

Figure 4. Summary of Wnt3a purification scheme

Top Panel) Flow chart of Wnt3a purification scheme. Middle Panel) SDS-PAGE analysis of samples obtained after each step of the purification procedure including dye-ligand chromatography pool 1 and pool 2 (Bp1 and Bp2), IMAC chromatography pool 1 and pool 2 (Ip1 and Ip2) and gel filtration chromatography of the IMAC pool 1 and pool 2 (GF1 and GF2). The gel was electrophoresed in the presence of β -mercaptoethanol and stained with Imperial protein stain. The last lane shows 2-mercaptoethanol-containing sample buffer alone. Molecular weight markers are shown at left. Bottom Panel) Densitometry scan of SDS-PAGE analysis of lanes GF1 and GF2 from Middle Panel.

Figure 5. Effect of Wnt3a on cellular levels of β-catenin

Mouse L cells were incubated with the indicated concentrations of Wnt3a for 3 h. Following incubation the cells were lysed and 10 µg lysate protein applied to a 10% SDS-PAGE gel. Separated proteins were transferred to PVDF membrane and, after blocking, probed with antibodies directed against β-catenin and GAPDH.

Figure 6. Effect of thrombin on isolated Wnt3a

Wnt3a (0.2 μ g) was incubated in the absence or presence of 0.2 μ g thrombin (10 μ l total volume) for 4 h at 37 $^{\circ}$ C. Following incubation the samples were subject to SDS-PAGE and stained with Imperial protein stain (left panel) or transferred to PVDF and probed with α -Wnt3a (right panel).

Table 1

Wnt3a Purification Flow Chart

	Absorbance, A ₂₈₀ Total ¹	Activity total U ²	Specific activity U/A ₂₈₀
Conditioned medium	27950	n. d.	n. d.
Dye-ligand, pool 1	14.4	2805 ± 112	195 ± 4
pool 2³	6.8	207 ± 23	30 ± 3
Cu²⁺ IMAC, pool 1	3.5	401 ± 18	116 ± 5
pool 2	0.8	504 ± 6	643 ± 8
Gel filtration 1	0.09	225 ± 8	2378 ± 83
Gel filtration 2	0.06	317 ± 31	5368 ± 519

¹ A₂₈₀ multiplied by volume, ml

² arbitrary units

³ pool was discarded

n.d., not determined

Table 2Effect of CHAPS replacement on Wnt3a solubility/biological activity¹

	CHAPS/TBS	MβCD/TBS	TBS
Yield, %	61.7 ± 3.4	16.8 ± 0.9	7.9 ± 0.9
Specific activity, %	100.0 ± 5.2	29.2 ± 9.9	1.7 ± 1.5
Stability			
Centrifugation², % remaining	88.7 ± 1.2	64.6 ± 2.0	9.9 ± 17.1
Flash freezing, % activity remaining	100 ± 16.3	36.5 ± 4.6	n.d.

¹ Values reported are the average of four measurements from two independent experiments. Details of the purification process, including a flow chart, electrophoresis analysis and densitometry can be found in Figure 4.

² 10,000 x g for 4 min at 4°C.

n.d. not detected