Published in final edited form as:

Arch Biochem Biophys. 2017 September 15; 630: 38–46. doi:10.1016/j.abb.2017.07.015.

Characterization of secondary structure and lipid binding behavior of N-terminal saposin like subdomain of human Wnt3a

Aparna Krishnamoorthy¹, Andrzej Witkowski³, Jesse J. Tran², Paul M.M. Weers², and Robert O. Ryan^{1,3,*}

¹Department of Nutritional Sciences and Toxicology, University of California Berkeley, Berkeley, California, USA 94720

²Department of Chemistry and Biochemistry, California State University, Long Beach, California, USA 90840

³Children's Hospital Oakland Research Institute, 5700 Martin Luther King Jr. Way, Oakland California, USA 94609

Abstract

Wnt signaling is essential for embryonic development and adult homeostasis in multicellular organisms. A conserved feature among Wnt family proteins is the presence of two structural domains. Within the N-terminal (NT) domain there exists a motif that is superimposable upon saposin-like protein (SAPLIP) family members. SAPLIPs are found in plants, microbes and animals and possess lipid surface seeking activity. To investigate the function of the Wnt3a saposin-like subdomain (SLD), recombinant SLD was studied in isolation. Bacterial expression of this Wnt fragment was achieved only when the core SLD included 82 NT residues of Wnt3a (NT-SLD). Unlike SAPLIPs, NT-SLD required the presence of detergent to achieve solubility at neutral pH. Deletion of two hairpin loop extensions present in NT-SLD, but not other SAPLIPs, had no effect on the solubility properties of NT-SLD. Far UV circular dichroism spectroscopy of NT-SLD yielded 50 to 60% α -helix secondary structure. Limited proteolysis of isolated NT-SLD in buffer and detergent micelles showed no differences in cleavage kinetics. Unlike prototypical saposins, NT-SLD exhibited weak membrane-binding affinity and lacked cell lytic activity. In cell-based canonical Wnt signaling assays, NT-SLD was unable to induce stabilization of β -catenin or modulate the extent of β -catenin stabilization induced by full-length Wnt3a. Taken together, the results indicate neighboring structural elements within full-length Wnt3a affect SLD conformational stability. Moreover, SLD function(s) in Wnt proteins appear to have evolved away from those commonly attributed to SAPLIP family members.

Graphical abstract

*Correspondence: Robert O. Ryan, robertryan@unr.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Structural model of *Xenopus* Wnt8 in complex with one the cysteine-rich domain of its co-receptor, frizzled 8 (grey space-fill). The saposin like domain within the overall Wnt structure is depicted in red.

Keywords

Canonical Wnt signal transduction; circular dichroism spectroscopy; limited proteolysis; liposomes; saposin; Wnt3a

Introduction

Evolutionally conserved Wnt proteins initiate a signaling cascade that is key to normal embryonic development and homeostasis throughout the adult life of metazoans [1]. In mammals, Wnts comprise a family of secreted ~350 amino acid, lipid-modified/ glycosylated, cysteine-rich proteins [2] that signal via canonical (β -catenin dependent) and non canonical (β -catenin independent) pathways [3]. In the well-characterized canonical pathway, Wnt engages the co-receptors “frizzled” (Fzd) and low density lipoprotein receptor related proteins 5 or 6 (LRP5/6), leading to stabilization of cytoplasmic β -catenin which migrates to the nucleus and functions as a transcriptional activator. Nuclear β -catenin co-activates the lymphoid enhancer binding factor/T cell specific transcription factor to regulate transcription of Wnt target genes. In the absence of Wnt, cytoplasmic β -catenin is phosphorylated, ubiquitinated and degraded by proteasomes [4]. Spatiotemporal expression of 19 different Wnt genes, 10 Fzd and 2 LRP give rise to diversity and complexity in mammalian Wnt signaling [5]. Thus, it is not surprising that dysregulation in Wnt signaling underlies diseases including cancer, metabolic syndrome and neurodegenerative disorders [6–9].

The X-ray crystal structure of *Xenopus* Wnt8 engaged with the cysteine-rich domain (CRD) of Fzd8 revealed two independently folded structural domains joined by a linker segment [10]. The N-terminal (NT) domain is comprised of six α -helices organized in a bundle. Extending from this core helix bundle are two narrow, elongated β -hairpins. The extreme tip of the first hairpin bears a conserved serine that serves as the site of posttranslational attachment of a palmitoleic acid moiety, which is required for intracellular trafficking and secretion of Wnt [11]. This monounsaturated fatty acid also contributes to Wnt8 interaction with Fzd8 CRD [10]. The smaller Wnt C-terminal (CT) domain adopts a long twisted β -hairpin reminiscent of a cysteine knot growth factor fold. As with hairpins in the NT domain, those in the CT domain are stabilized by a series of disulfide bonds [10,12].

Based on structural analysis, a discrete region within the NT domain of Wnt8 was identified as resembling a saposin-like protein (SAPLIP) fold [13,14]. Prototypical SAPLIPs exist either as independently folded proteins or subdomains within larger protein structures. SAPLIPs consist of a bundle of 4–5 amphipathic α -helices with 3 intra-domain disulfide bonds [15]. It has been postulated that, when presented with an appropriate lipid surface, the SAPLIP helix bundle unfurls, exposing a hydrophobic interior that interacts with lipid surfaces [16,17]. This conformational flexibility facilitates the biological functions of SAPLIPs including membrane interaction, pore formation and/or cell lysis [18–20]. In fact, SAPLIPs encompass a large protein family that manifest diverse functions including pulmonary surface tension regulation, antimicrobial activity, eukaryotic cell lysis and/or cofactor for sphingolipid degradative enzymes [15,21–25].

The identification of a protein fold resembling SAPLIPs as a structural element within the NT domain of Wnt family members raises the possibility that Wnts adopt alternate conformational states via their saposin-like subdomain (SLD). The hypothesis that Wnt3a-SLD possesses functions ascribed to SAPLIP family members was examined in the present study. Constructs encoding SLD alone, SLD plus 82 NT residues (NT-SLD) and an NT-SLD lacking two Wnt-specific β -hairpins (NT-SLD(nh)) were produced in *E. coli*. Results obtained indicate SLD expression is dramatically improved when 82 NT residues of Wnt3a are attached to the core SLD sequence. Furthermore, although β -hairpin deleted SLD more closely resembles prototypical SAPLIPs, no differences in protein expression level or solubility properties were observed. Overall, the data suggest under the conditions examined, Wnt3a SLD has evolved to rely on other elements of the Wnt structure to maintain a stable fold and does not manifest classical functions associated with SAPLIPs in isolation.

Materials and Methods

Chemicals and reagents

Bacterial growth medium, dodecyltrimethylammonium chloride (DTAC), sodium dodecyl sulfate (SDS), reduced and oxidized glutathione were from Sigma-Aldrich. Dithiothreitol (DTT) was from Thermo Fisher Scientific. Thrombin was from Cayman Chemical. 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was purchased from A.G. Scientific Inc. Oligonucleotide primers were from Elim Oligo. Murine Wnt3a was expressed in stably transfected *Drosophila* S2 cells and isolated from conditioned media

according to [26]. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin and Halt Protease Inhibitor cocktail were obtained from Thermo Fisher Scientific. Precast 4–20% and 10% acrylamide slab gels were from Bio-Rad Laboratories. Mouse embryonic fibroblast L cells were provided by Dr. Roel Nusse [27]. 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was purchased from Avanti Polar Lipids Inc. Gel Code Blue and Imperial Protein stain was from Thermo Scientific. CellTiter 96 Non-Radioactive Cell Proliferation (MTT; 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay kit was from Promega. Prestained Precision Plus protein standards were purchased from BioRad. Mouse anti- β -catenin antibody was purchased from BD Transduction Laboratories and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from Millipore. Horseradish Peroxidase (HRP)-conjugated anti mouse secondary antibody was from Vector Labs and WesternBright ECL HRP substrate was from Advanta.

Expression vector construction

A cDNA encoding human *WNT3A* (Transomic Technologies) served as template for amplification of a 420 bp sequence corresponding to the SLD using the following primers: FwdSLDNde1 (5' - gggcccatatggtgctggac - 3') and RevSLDXho1 (5' - ctccaccactcgagcatctccga - 3'). The same human *WNT3A* template was used to amplify a 666 bp sequence (NT-SLD) using FwdNTSLDNde1 (5' -atcatatgagctacccgatctggtgctcg - 3') and RevSLDXho1. Finally, a 531 bp cDNA SLD construct missing the 2 β -hairpin extensions NT-SLD no hairpins(nh) was custom ordered from GenScript. All 3 constructs were ligated into a pET22b plasmid vector (Novagen) at Nde1 and Xho1 (New England Biolabs). Each construct was sequence verified and transformed into SHuffle T7 Express *E. coli* cells (New England Biolabs) to facilitate disulfide bond formation [28].

Recombinant protein production and purification

Bacteria were cultured in NZCYM media plus ampicillin (50 μ g/ml) at 30 °C. When the OD₆₀₀ reached 0.6, SLD synthesis was induced by the addition of isopropyl β -D-1-thiogalactopyranoside (1 mM final concentration). Following overnight culture, bacteria were pelleted by centrifugation, re-suspended in 50 mM Tris-HCl, pH 7.2, 0.1 M NaCl, 0.1 mM EDTA, 1 mM DTT and disrupted by probe sonication. Preliminary expression studies revealed that NT-SLD and NT-SLD(nh) localized to inclusion bodies. Subsequently, *E. coli* cells were processed according to Burgess [29]. Briefly, Triton X-100 (1% final concentration) was added to a sonicated cell lysate, incubated on ice for 10 min and centrifuged at 20,000g for 15 min. This process was repeated at least 6 times or until a white pellet appeared. The precipitate was re-suspended in buffer without Triton X-100 and centrifuged at 20,000g for 15 min to pellet inclusion bodies. The washed pellet was re-suspended in 50 mM Tris-HCl, pH 7.2 containing 6 M guanidine (Gdn HCl, 0.1 M NaCl, 0.1 mM DTT) and incubated at 42–50 °C to dissolve the pellet. Following this, the sample was centrifuged at 20,000g for 15 min. The supernatant was filtered (0.22 μ m) to remove particulate matter and applied to a Hi-Trap affinity column (GE Healthcare) as per the manufacturer's instructions. Bound protein was eluted in 20 mM Tris-HCl, pH 7.2, 0.5 M NaCl, 0.5 M imidazole, 6 M Gdn HCl, dialyzed against H₂O, lyophilized and stored at –20 °C.

Recombinant protein solubilization

Lyophilized protein was re-suspended (4 mg/ml) in 2% DTAC (w/v) in the presence of 2 μ M oxidized glutathione and 10 μ M reduced glutathione [30]. The sample was “dilution-folded” in 10 mM sodium phosphate, pH 7, 0.6 M arginine HCl to a final protein concentration of ~0.02 mg/ml, thereby lowering the DTAC concentration below its critical micelle concentration (~30 mM) [31]. Subsequently, the sample was concentrated with an Amicon stirred cell fitted with a 10K molecular weight cutoff membrane and dialyzed against 10 mM Na phosphate, pH 7, 0.6 M arginine HCl to remove residual DTAC. Protein concentrations were determined by the Bicinchoninic acid assay (Thermo Fisher Scientific) and purity assessed by SDS-polyacrylamide gel electrophoresis (PAGE). In some cases, sample protein content was determined spectrophotometrically (280 nm) using an extinction coefficient of 45210 M⁻¹ cm⁻¹ for NT-SLD and 22710 M⁻¹ cm⁻¹ for NT-SLD(nh).

Far UV circular dichroism spectroscopy

Far UV circular dichroism (CD) spectroscopy measurements were performed on a Jasco 810 spectropolarimeter. Scans were collected thrice to obtain an average value, using a 0.1 cm path length and 0.2 mg/ml protein concentration. For low pH measurements, NT-SLD was dissolved in 50 mM Na citrate, 20 mM NaCl, pH 3 and measured in the presence of 1% SDS and 50% trifluoroethanol. In another set of experiments, NT-SLD or NT-SLD(nh) were dissolved in 2% DTAC and spectra collected in the presence of 20 mM Na phosphate, pH 7.2, 1mM DTT. Ellipticity values were converted into molar ellipticity (degrees cm² dmol⁻¹) using mean residue weight values of 25,035 Da for NT-SLD (228 residues) and 20,127 Da for NT-SLD(nh) (185 residues). Protein secondary structure content was calculated using CDSSTR, a variable selection method and SMP180 reference set through DichroWeb [32–34].

Limited proteolysis

NT-SLD and NT-SLD(nh) were incubated in the presence and absence of thrombin (Sigma-Aldrich) at a 1:3 thrombin : SLD protein ratio for 1, 2 and 4 h at 37 °C in 10 mM Na phosphate, pH 7, 0.6 M arginine HCl in the presence or absence of 1% CHAPS (w/v). Following incubation, proteins were separated by SDS-PAGE and visualized with Imperial protein stain.

Liposome preparation

Ten mg DMPC in chloroform was dried under N₂ gas in a 10 ml round bottom flask and dried further under vacuum overnight. The dried lipid film was dispersed in 500 μ l 10 mM Na phosphate, pH 7, 0.6 M arginine HCl and vortexed. The lipid suspension was extruded with 21 passes through a 100 nm polycarbonate membrane in a Thermobarrel extruder maintained at 30–37 °C. The resulting liposome preparation was incubated with NT-SLD or NT-SLD(nh) at 37 °C for 1 h (20:1 liposome to protein ratio; 2 mg liposomes and 100 μ g protein in a final volume = 200 μ l). Following incubation, liposome-associated SLDs were separated from free SLDs by sucrose gradient centrifugation. Samples were mixed with an equal volume of 80% sucrose and transferred to a centrifuge tube. The 40% sucrose solution was topped with a 20% sucrose solution followed by an 8% sucrose solution. The tube

contents were then centrifuged at 200,000g for 3 h at 4 °C. Following centrifugation, fractions (200 µl) were collected from the top and analyzed for liposome content turbidimetrically (310 nm) and protein content by SDS-PAGE.

Fluorescence spectroscopy

Specified SLDs were excited at 280 nm and emission recorded between 300 and 450 nm (2 nm slit width) on a Horiba Jobin Yvon FluoroMax-4 luminescence spectrometer. Spectra were obtained in 10 mM Na phosphate, pH 7, 0.6 M arginine HCl in the presence and absence of 2% DTAC.

Biological membrane lytic activity

L cells (6000 cells/well, 96-well plate) were cultured in high glucose DMEM containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin overnight at 37 °C. After 24 h, the cells were replenished with fresh medium supplemented with specified concentrations of NT-SLD or NT-SLD(nh). After 24 h incubation with SLDs, cell viability was measured with MTT assay.

Canonical Wnt3a signaling activity

L cells (0.5×10^6 cells/well, 12-well plate) were incubated with specified amounts of NT-SLD or NT-SLD(nh) for 3 h at 37 °C and harvested using RIPA cell lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100) containing 1X Halt protease inhibitor cocktail mix. The protein concentration of cell lysates was measured and 10 µg aliquots heated to 95 °C for 10 min in the presence of SDS reducing buffer, applied to precast 10% polyacrylamide gels and electrophoresed at 120 V. Separated proteins were transferred to a polyvinylidene difluoride membrane, blocked in 5% BSA and probed with mouse anti-β-catenin (1:2,000 dilution) and mouse anti-GAPDH (1:2,500 dilution) monoclonal antibodies. Positive bands were detected with HRP-conjugated anti-mouse secondary antibody (1:5,000 dilution). Assays were performed in triplicate.

Protein Bioinformatics

The three dimensional structure of *Xenopus* Wnt8 in complex with Fz8 CRD [10] was analyzed *in silico* using Research Collaboratory for Structural Bioinformatics Protein Data Bank entry (PDB: 4FOA).

Results

Bacterial expression of recombinant Wnt3a SLD

To study the Wnt SLD motif in isolation, a plasmid vector encoding amino acids 83-VLDK...ASEM-222 of mature human Wnt3a was transformed into three different strains of *E. coli* (Rosetta, BL21 and SHuffle T7). Following induction with IPTG, SLD production was examined at various temperatures ranging from 16°C to 37°C and for time periods ranging from 4 h to 24 h. After each culture period, bacterial supernatant, cytosol and inclusion bodies were separated and compared to control cultures for SLD production. Despite several attempts, no SLD expression was detected in the bacterial supernatant or

pellet fractions. Computer assisted analysis of predicted α -helix segments in the SLD motif revealed helix 'a' is mostly hydrophobic while helices 'b', 'c' and 'd' are amphipathic, similar to the helix pattern found in SAPLIPs [35]. 3D view analysis of the *Xenopus* Wnt8 crystal structure provided evidence that residues in the extreme N-terminus of the protein (Figure 1A) orient perpendicular to the core SLD 4-helix bundle in a "protective" belt-like manner that shields helix 'a'. Based on this, the original SLD construct was modified to include 82 NT residues from Wnt3a (Figure 1B). Unlike the original SLD construct, robust expression of NT-SLD (residues 1-SYPI...ASEM-222) was observed, with large amounts of recombinant protein accumulating in inclusion bodies. To better mimic the structure of SAPLIPs, a third construct was generated wherein two hairpin extensions present in Wnt SLDs but not in other saposin family proteins, were deleted (residues 112 to 141 comprising hairpin 1 were replaced by the amino acid sequence Gly-Ala-Gly while residues 178 to 205 comprising hairpin 2 were replaced by Gly-Ala-Gly-Ala-Gly). The resulting construct, NT-SLD(nh), expressed well in *E. coli*, with the protein product accumulating in inclusion bodies.

Solution properties of recombinant Wnt3a-NT-SLDs

The relative purity of isolated NT-SLD and NT-SLD(nh) were assessed by SDS-PAGE (Figure 2). Although isolated NT-SLD was fully soluble in 50 mM sodium citrate, pH 3, 150 mM NaCl, it was insoluble in neutral pH buffers. However, solubility was conferred to NT-SLD at neutral pH when DTAC (2% w/v) was introduced. Subsequent removal of DTAC by dilution and dialysis against 10 mM sodium phosphate pH 7, 0.6 M arginine HCl retained NT-SLD solubility at neutral pH in the absence of detergent. To determine the effect of hairpin deletion on NT-SLD solubility, NT-SLD(nh) was purified and isolated from inclusion bodies using the same process. This ~20 kDa protein was soluble at pH 3 and also required the presence of 0.6 M arginine HCl to retain solubility in neutral pH buffer upon removal of detergent.

Far UV CD spectroscopy of SLDs

To evaluate the secondary structure content of NT-SLD and NT-SLD(nh), far UV CD spectra were collected. NT-SLD in 50 mM Na citrate, pH 3, 20 mM NaCl yielded a spectrum with little negative ellipticity. Upon addition of 1% SDS or 50% trifluoroethanol, however, distinct minima appeared at 208 nm and 222 nm, consistent with induction of α -helix secondary structure (Figure 3). At neutral pH (10 mM sodium phosphate, pH 7, 0.6 M arginine HCl) the absorption properties of L-arginine interfered with the SLD signal. Subsequently, spectra of NT-SLD and NT-SLD(nh) were collected in 20 mM sodium phosphate, pH 7.2, 2% DTAC. The spectra displayed strong minima at 208 and 222 nm and secondary structure calculations indicated 49% α -helix for NT-SLD and 63% for NT-SLD(nh). Inclusion of oxidized/reduced glutathione and DTT had no effect on the secondary structure content of NT-SLD or NT-SLD(nh).

SLD interaction with liposomes

To assess their membrane interaction properties, NT-SLD and NT-SLD(nh) were incubated with liposomes. Following incubation, lipid-bound and free SLDs were separated by sucrose gradient centrifugation, fractionated and analyzed (Figure 4). Turbidimetric measurement of

liposome light scatter intensity revealed that, under all incubation conditions, liposomes migrate predominantly to top gradient fractions 2 and 3 while bottom fractions 4 and 5 were essentially free of them. On the other hand, in the absence of liposomes, NT-SLD and NT-SLD(nh) were recovered in fractions 4 and 5. Upon incubation with liposomes, although the bulk of the NT-SLD and NT-SLD(nh) were recovered in fractions 4 and 5, a small portion of these proteins were found in lower density, liposome-containing fractions.

Fluorescence analysis of SLD-detergent micelle interaction

Given that NT-SLD exhibited limited binding to liposomes yet was stabilized in the presence of detergent micelles, the effect of DTAC on the intrinsic fluorescence of SLDs was examined (Figure 5). Fluorescence emission spectra were collected for each SLD in the absence and presence of 2% DTAC. NT-SLD has seven Trp residues (positions 5, 6, 68, 132, 134, 199 and 200) while NT-SLD(nh) has three (positions 5, 6, 68). In the absence of DTAC, normalized fluorescence emission spectra (excitation 280 nm) of NT-SLD and NT-SLD(nh) gave rise to wavelengths of maximum fluorescence emission of 346 nm and 341 nm, respectively. In the presence of DTAC, a ~2.5 fold increase in quantum yield was recorded for NT-SLD and NT-SLD(nh), with no shift in the wavelength of maximum fluorescence emission.

Limited proteolysis of SLDs

To probe whether binding to detergent micelles induces a conformational change in NT-SLD or NT-SLD(nh) similar to that reported for SAPLIPs [36], limited proteolysis experiments were performed. Based on the previously reported thrombin sensitivity of Wnt3a [26] and its binding to CHAPS micelles [37], thrombolytic cleavage of SLDs was evaluated in the absence and presence of CHAPS. Whereas thrombin prefers to cleave Arg-Gly sequences [38], the SLDs under investigation lack this sequence element. However, NT-SLD and NT-SLD(nh) contain an Arg-Ser sequence that is sensitive to thrombin digestion [39]. Under the conditions employed, a time-dependent increase in NT-SLD and NT-SLD(nh) proteolysis was observed, as judged by substrate reduction/product accumulation over the course of 4 h (Figure 6). The proteolytic fragments generated from NT-SLD were 17 kDa and 7 kDa in size while those from NT-SLD(nh) were 14 kDa and 5 kDa. Cleavage of NT-SLD(nh) was monitored by the appearance of the 14 kDa fragment since the 5 kDa fragment was not detected under these electrophoresis conditions. No significant differences in thrombin sensitivity were observed between NT-SLD and NT-SLD(nh) in the presence or absence of CHAPS micelles.

Effect of NT-SLDs on cell membrane lysis

A known property of certain SAPLIPs is cell membrane lysis [25,40]. To determine if NT-SLD or NT-SLD(nh) possess cell lytic activity, the proteins were incubated with cultured fibroblasts. Following incubation with varying concentrations of NT-SLD or NT-SLD(nh), cell viability was assayed (Figure 7). Compared to buffer treated control cells, no differences in cell viability were detected in cells incubated with either protein.

Wnt3a canonical signaling in the presence of NT-SLDs

To assess effect of NT-SLD or NT-SLD(nh) on canonical Wnt3a signaling activity, cultured fibroblasts were treated with increasing concentrations of NT-SLD or NT-SLD(nh) followed by determination of cellular β -catenin levels (a measure of canonical Wnt signaling; [26]). Similar to untreated control cells, β -catenin levels were below the limits of detection following incubation with either NT-SLD or NT-SLD(nh) (Figure 8). By contrast, levels of β -catenin increased dramatically in response to treatment with 60 ng/ml full-length Wnt3a. This level of activation was unaffected by inclusion of increasing concentrations of NT-SLD or NT-SLD(nh).

Discussion

The Wnt protein structure provides valuable information pertinent to its post-secretory migration via diffusion, and/or interaction with carriers, that ultimately leads to engagement of cell surface receptors and manifestation of biological activity [41,42]. At the target cell surface, bipartite binding of Wnt's globular NT domain and CT cytokine-like domain to Fzd CRD has led to the hypothesis that, during evolution of Wnts, a gene fusion event occurred to create its two domain structure [13,43]. Such an event may have included a SAPLIP gene, which became integrated into the molecular architecture of Wnt. Analysis of *Xenopus* Wnt8 X-ray structure reveals the presence of a saposin-like subdomain as a structural feature of Wnt [13]. If so, has this domain retained or lost functional properties associated with SAPLIP family members? To address this, a reductionist approach was employed to evaluate the functional properties of this subdomain in isolation. Recombinant Wnt SLD was studied in the absence of posttranslational modifications using bacterial expression system, since SAPLIP family members lack posttranslational modifications. During initial experiments performed to determine the feasibility of bacterial expression of human Wnt3a SLD the expression construct included only those residues identified as being part of the saposin structure. However, little or no SLD protein was produced. On the other hand, when the construct was expanded to include 82 NT amino acids of human Wnt3a, protein production greatly increased. The reason for this is unclear but may be related to a potential structural role imparted by these 82 residues. In the X-ray structure of *Xenopus* Wnt8 these residues adopt an α -helix that contacts helix 'a' of SLD. Thus, it is conceivable that Wnt SLD is unstable in the absence of this region of the Wnt protein. Another feature unique to Wnt SLD is the presence of two elongated hairpins that extend from the SLD. Given that these structural features are not present in SAPLIPs, a construct was generated in which these elements were deleted. Among 3 different plasmid constructs generated, SLD, NT-SLD and NT-SLD(nh), the latter two led to recombinant protein production upon transformation into *E. coli*. The expressed proteins were isolated in mg quantities, permitting their structural and functional properties to be investigated.

To our knowledge, the present study is the first investigation into the molecular properties of an isolated Wnt subdomain in which structural and biochemical analyses were used to evaluate its potential functionality in the context of full-length Wnt. Based on secondary structure analysis, it was observed that, similar to other SAPLIP family members [44,45], isolated NT-SLD and NT-SLD(nh) adopt α -helix secondary structure in aqueous media,

albeit only under special circumstances. Although both of the SLDs studied were soluble in low pH buffer, they lack secondary structure under this condition. This property is unlike many SAPLIPs, which are known to retain secondary structure and function at low pH [25]. These results suggest additional structural elements within full-length Wnt3a affect the structural integrity of the SLD subdomain.

A general feature of SAPLIPs is the presence of a loosely packed helix bundle core that is predicted to facilitate conformational opening and closing in response to pH change or exposure to lipid [15,36]. The presence of three intramolecular disulfide bonds in SAPLIPs may define the limits of conformational flexibility and dictate the route of helix bundle opening and closing. As a result, members of the SAPLIP protein family interact reversibly with membranes, either at cell surface or an organelle to facilitate cell lysis, lipid modifying enzyme activity or transport processes [25,46]. While the SLD of Wnt also possesses a loosely packed core, corresponding disulfide bonds are lacking, such that fewer restrictions exist to limit conformational flexibility [10,14]. Although SLDs interact with detergent micelles, classic helix bundle opening to engage the membrane surface of liposomes did not occur to a significant extent.

Further information about SLD conformational flexibility and changes in protein microenvironment was obtained in studies with detergent micelles using fluorescence spectroscopy. Although the wavelength of maximum Trp fluorescence emission did not shift upon SLD binding to micelles, a large enhancement in quantum yield was noted. The increase in quantum yield is indicative of SLD binding to the micelle surface while the location of SLD Trp residues outside the core helix bundle structure may underlie the absence of a blue shift in Trp fluorescence emission maximum upon micelle binding.

In an effort to monitor changes in conformation upon SLD interaction with micelles, thrombin cleavage sensitivity studies were performed. Previous studies revealed thrombin recognizes an Arg-Ser sequence element in Wnt3a as a cleavage site [26]. Thus, we hypothesized that, if this region of the protein changes its orientation and exposure to solvent, differences in thrombin cleavage sensitivity may be observed in the presence/absence of detergent micelles. The results obtained indicate that micelle interaction did not confer protection from thrombolytic cleavage as compared to the same SLDs in aqueous media. This may be explained by the location of the Arg-Ser sequence near one end of the SLD, a region that is not predicted to make intimate contact with the micelle surface. More information may be gained by examining the cleavage sensitivity of SLD variants in which the cleavage site is relocated within the protein sequence.

Among Wnt family protein members, *Drosophila* WntD is unique in that it is neither glycosylated nor fatty acylated yet it signals through the Fzd4 receptor to affect patterning, adult immune responses and primordial germ cells migration to gonads [14]. Similarly, recombinant NT-SLD and NT-SLD(nh) studied herein lack fatty acylation. Given the binding interaction identified between the fatty acyl chain on *Xenopus* Wnt8 and Fzd8 CRD by X-ray crystallography [10], it is possible, if not likely, that the lack of a fatty acyl group in SLDs generated in this study interferes with their ability to modulate canonical Wnt signaling activity. The finding that SLDs did not interfere or enhance the ability of full

length Wnt3a to induce β -catenin stabilization suggests these proteins do not interact with one another. Future studies will be required to determine the effect of post-translational SLD fatty acylation on canonical Wnt signaling activity as well as any effects on manifestation of SAPLIP-like properties such as liposome binding of lipid-modified SLD. Such studies would reveal if fatty acylated SLD alone could accentuate or interfere with Wnt signaling. Based on the findings of the present study, the evidence suggests that Wnt3a SLD has deviated from ancestral SAPLIPs such that, while maintaining the overall protein fold, it serves a predominantly structural role in the broader context of Wnt proteins.

Acknowledgments

This work was supported by a grant from the US National Institutes of Health (R37 HL64159 and GM089564). The authors thank Dr. Giorgio Cavigiolio for use of the FluoroMax-4 luminescence spectrometer.

Abbreviations

NT	N-terminal
CT	C-terminal
SAPLIP	saposin-like protein
SLD	saposin-like subdomain
SLD	NT-SLD(nh), N-terminal saposin-like subdomain no hairpins
CRD	cysteine-rich domain
Fzd	frizzled
LRP5/6	low density lipoprotein receptor related proteins 5 or 6
DTAC	dodecyltrimethylammonium chloride
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
DMEM	Dulbecco's Modified Eagle's Medium
FBS	fetal bovine serum
HRP	horseradish peroxidase
DTT	dithiothreitol
Gdn HCl	guanidine hydrochloride
SDS	sodium dodecyl sulfate
CD	circular dichroism

SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis

GAPDH glyceraldehyde-3-phosphate dehydrogenase

References

1. Nusse R. Wnt signaling and stem cell control. *Cell Res.* 2008; 18:523–527. DOI: 10.1038/cr.2008.47 [PubMed: 18392048]
2. Holstein TW. The evolution of the Wnt pathway. *Cold Spring Harb Perspect Biol.* 2012; 4:a007922.doi: 10.1101/cshperspect.a007922 [PubMed: 22751150]
3. Staal FJT, Luis TC, Tiemessen MM. WNT signalling in the immune system: WNT is spreading its wings. *Nat Rev Immunol.* 2008; 8:581–593. DOI: 10.1038/nri2360 [PubMed: 18617885]
4. Schulte G. Frizzleds and WNT/ β -catenin signaling – The black box of ligand–receptor selectivity, complex stoichiometry and activation kinetics. *Eur J Pharmacol.* 2015; 763:191–195. DOI: 10.1016/j.ejphar.2015.05.031 [PubMed: 26003275]
5. Angers S, Moon RT. Proximal events in Wnt signal transduction. *Nat Rev Mol Cell Biol.* 2009; 10:468.doi: 10.1038/nrm2717 [PubMed: 19536106]
6. Moon RT, Kohn AD, Ferrari GV De, Kaykas A. WNT and β -catenin signalling: diseases and therapies. *Nat Rev Genet.* 2004; 5:691–701. DOI: 10.1038/nrg1427 [PubMed: 15372092]
7. Schinner S. Wnt-signalling and the metabolic syndrome. *Horm Metab Res.* 2009; 41:159–63. DOI: 10.1055/s-0028-1119408 [PubMed: 19214925]
8. Berwick DC, Harvey K. The importance of Wnt signalling for neurodegeneration in Parkinson's disease. *Biochem Soc Trans.* 2012; 40:1123–8. DOI: 10.1042/BST20120122 [PubMed: 22988876]
9. Libro R, Bramanti P, Mazzon E. The role of the Wnt canonical signaling in neurodegenerative diseases. *Life Sci.* 2016; 158:78–88. DOI: 10.1016/j.lfs.2016.06.024 [PubMed: 27370940]
10. Janda CY, Waghray D, Levin AM, Thomas C, Garcia KC. Structural basis of Wnt recognition by Frizzled. *Science.* 2012; 337:59–64. DOI: 10.1126/science.1222879 [PubMed: 22653731]
11. Takada R, Satomi Y, Kurata T, Ueno N, Norioka S, Kondoh H, Takao T, Takada S. Monounsaturated fatty acid modification of Wnt protein: its role in Wnt secretion. *Dev Cell.* 2006; 11:791–801. DOI: 10.1016/j.devcel.2006.10.003 [PubMed: 17141155]
12. Kumar S, Žigman M, Patel TR, Trageser B, Gross JC, Rahm K, Boutros M, Gradl D, Steinbeisser H, Holstein T, Stetefeld J, Özbek S. Molecular dissection of Wnt3a-Frizzled8 interaction reveals essential and modulatory determinants of Wnt signaling activity. *BMC Biol.* 2014; 12:44.doi: 10.1186/1741-7007-12-44 [PubMed: 24885675]
13. Bazan JF, Janda CY, Garcia KC. Structural architecture and functional evolution of Wnts. *Dev Cell.* 2012; 23:227–32. DOI: 10.1016/j.devcel.2012.07.011 [PubMed: 22898770]
14. Chu MLH, Ahn VE, Choi HJ, Daniels DL, Nusse R, Weis WI. Structural Studies of Wnts and identification of an LRP6 binding site. *Structure.* 2013; 21:1235–42. DOI: 10.1016/j.str.2013.05.006 [PubMed: 23791946]
15. Anderson DH, Sawaya MR, Cascio D, Ernst W, Modlin R, Krensky A, Eisenberg D. Granulysin crystal structure and a structure-derived lytic mechanism. *J Mol Biol.* 2003; 325:355–65. [PubMed: 12488100]
16. Munford RS, Sheppard PO, O'Hara PJ. Saposin-like proteins (SAPLIP) carry out diverse functions on a common backbone structure. *J Lipid Res.* 1995; 36:1653–63. [PubMed: 7595087]
17. Bruhn H. A short guided tour through functional and structural features of saposin-like proteins. *Biochem J.* 2005; 389:249–57. DOI: 10.1042/BJ20050051 [PubMed: 15992358]
18. Michalek M, Leippe M. Mechanistic Insights into the Lipid Interaction of an Ancient Saposin-like Protein. *Biochemistry.* 2015; 54:1778–1786. DOI: 10.1021/acs.biochem.5b00094 [PubMed: 25715682]
19. Rossmann M, Schultz-Heienbrok R, Behlke J, Rimmel N, Alings C, Sandhoff K, Saenger W, Maier T. Crystal structures of human saposins C and D: implications for lipid recognition and membrane interactions. *Structure.* 2008; 16:809–17. DOI: 10.1016/j.str.2008.02.016 [PubMed: 18462685]

20. Winkelmann J, Leippe M, Bruhn H. A novel saposin-like protein of *Entamoeba histolytica* with membrane-fusogenic activity. *Mol Biochem Parasitol.* 2006; 147:85–94. DOI: 10.1016/j.molbiopara.2006.01.010 [PubMed: 16529828]
21. Liepinsh E, Andersson M, Ruyschaert JM, Otting G. Saposin fold revealed by the NMR structure of NK-lysin. *Nat Struct Biol.* 1997; 4:793–5. [PubMed: 9334742]
22. Piatigorsky J, Norman B, Dishaw LJ, Kos L, Horwitz J, Steinbach PJ, Kozmik Z. J3-crystallin of the jellyfish lens: similarity to saposins. *Proc Natl Acad Sci U S A.* 2001; 98:12362–7. DOI: 10.1073/pnas.231310698 [PubMed: 11675486]
23. Bornhauser BC, Olsson P-A, Lindholm D. MSAP Is a Novel MIR-interacting Protein That Enhances Neurite Outgrowth and Increases Myosin Regulatory Light Chain. *J Biol Chem.* 2003; 278:35412–35420. DOI: 10.1074/jbc.M306271200 [PubMed: 12826659]
24. Hecht O, Van Nuland NA, Schleinkofer K, Dingley AJ, Bruhn H, Leippe M, Grötzinger J. Solution structure of the pore-forming protein of *Entamoeba histolytica*. *J Biol Chem.* 2004; 279:17834–41. DOI: 10.1074/jbc.M312978200 [PubMed: 14970207]
25. Yang L, Johansson J, Ridsdale R, Willander H, Fitzen M, Akinbi HT, Weaver TE. Surfactant Protein B Propeptide Contains a Saposin-Like Protein Domain with Antimicrobial Activity at Low pH. *J Immunol.* 2010; 184:975–983. DOI: 10.4049/jimmunol.0900650 [PubMed: 20007532]
26. Witkowski A, Krishnamoorthy A, Su B, Beckstead JA, Ryan RO. Isolation and characterization of recombinant murine Wnt3a. *Protein Expr Purif.* 2015; 106:41–48. DOI: 10.1016/j.pep.2014.10.015 [PubMed: 25448592]
27. Willert K, Brown JD, Danenberg E, Duncan AW, Weissman IL, Reya T, Yates JR, Nusse R. Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature.* 2003; 423:448–52. DOI: 10.1038/nature01611 [PubMed: 12717451]
28. Kong B, Guo GL. Soluble expression of disulfide bond containing proteins FGF15 and FGF19 in the cytoplasm of *Escherichia coli*. *PLoS One.* 2014; 9:e85890.doi: 10.1371/journal.pone.0085890 [PubMed: 24465767]
29. Burgess RR. Chapter 17 Refolding Solubilized Inclusion Body Proteins. in: *Methods Enzymol.* 2009; :259–282. DOI: 10.1016/S0076-6879(09)63017-2
30. Kudou M, Yumioka R, Ejima D, Arakawa T, Tsumoto K. A novel protein refolding system using lauroyl-L-glutamate as a solubilizing detergent and arginine as a folding assisting agent. *Protein Expr Purif.* 2011; 75:46–54. DOI: 10.1016/j.pep.2010.08.011 [PubMed: 20817098]
31. Oviedo-Roa R, Martínez-Magadán JM, Muñoz-Colunga A, Gómez-Balderas R, Pons-Jiménez M, Zamudio-Rivera LS. Critical micelle concentration of an ammonium salt through DPD simulations using COSMO-RS-based interaction parameters. *AIChE J.* 2013; 59:4413–4423. DOI: 10.1002/aic.14158
32. Abdul-Gader A, Miles AJ, Wallace BA. A reference dataset for the analyses of membrane protein secondary structures and transmembrane residues using circular dichroism spectroscopy. *Bioinformatics.* 2011; 27:1630–6. DOI: 10.1093/bioinformatics/btr234 [PubMed: 21505036]
33. Whitmore L, Wallace BA. Protein secondary structure analyses from circular dichroism spectroscopy: Methods and reference databases. *Biopolymers.* 2008; 89:392–400. DOI: 10.1002/bip.20853 [PubMed: 17896349]
34. Sreerama N, Woody RW. Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set. *Anal Biochem.* 2000; 287:252–60. DOI: 10.1006/abio.2000.4880 [PubMed: 11112271]
35. Qi X, Grabowski GA. Differential membrane interactions of saposins A and C: Implications for the functional specificity. *J Biol Chem.* 2001; 276:27010–27017. DOI: 10.1074/jbc.M101075200 [PubMed: 11356836]
36. Popovic K, Holyoake J, Pomès R, Privé GG. Structure of saposin A lipoprotein discs. *Proc Natl Acad Sci U S A.* 2012; 109:2908–12. DOI: 10.1073/pnas.1115743109 [PubMed: 22308394]
37. Lalefar NR, Witkowski A, Simonsen JB, Ryan RO. Wnt3a nanodisks promote ex vivo expansion of hematopoietic stem and progenitor cells. *J Nanobiotechnology.* 2016; 14:66.doi: 10.1186/s12951-016-0218-5 [PubMed: 27553039]
38. Waugh DS. An overview of enzymatic reagents for the removal of affinity tags. *Protein Expr Purif.* 2011; 80:283–93. DOI: 10.1016/j.pep.2011.08.005 [PubMed: 21871965]

39. Le Bonniec BF, Myles T, Johnson T, Knight CG, Tapparelli C, Stone SR. Characterization of the P2' and P3' specificities of thrombin using fluorescence-quenched substrates and mapping of the subsites by mutagenesis. *Biochemistry*. 1996; 35:7114–22. DOI: 10.1021/bi952701s [PubMed: 8679538]
40. Bryksa BC, Bhaumik P, Magracheva E, De Moura DC, Kurylowicz M, Zdanov A, Dutcher JR, Wlodawer A, Yada RY. Structure and mechanism of the saposin-like domain of a plant aspartic protease. *J Biol Chem*. 2011; 286:28265–75. DOI: 10.1074/jbc.M111.252619 [PubMed: 21676875]
41. Miyabayashi T, Teo JL, Yamamoto M, McMillan M, Nguyen C, Kahn M. Wnt/beta-catenin/CBP signaling maintains long-term murine embryonic stem cell pluripotency. *Proc Natl Acad Sci U S A*. 2007; 104:5668–73. DOI: 10.1073/pnas.0701331104 [PubMed: 17372190]
42. Nusse R, Varmus H. Three decades of Wnts: a personal perspective on how a scientific field developed. *EMBO J*. 2012; 31:2670–84. DOI: 10.1038/emboj.2012.146 [PubMed: 22617420]
43. MacDonald BT, Hien A, Zhang X, Iranloye O, Virshup DM, Waterman ML, He X. Disulfide bond requirements for active Wnt ligands. *J Biol Chem*. 2014; 289:18122–36. DOI: 10.1074/jbc.M114.575027 [PubMed: 24841207]
44. Egas C, Lavoura N, Resende R, Brito RM, Pires E, de Lima MC, Faro C. The saposin-like domain of the plant aspartic proteinase precursor is a potent inducer of vesicle leakage. *J Biol Chem*. 2000; 275:38190–6. DOI: 10.1074/jbc.M006093200 [PubMed: 10982803]
45. Waring AJ, Chen Y, Faull KF, Stevens R, Sherman MA, Fluharty AL. Porcine Cerebroside Sulfate Activator (Saposin B) Secondary Structure: CD, FTIR, and NMR Studies. *Mol Genet Metab*. 1998; 63:14–25. DOI: 10.1006/mgme.1997.2646 [PubMed: 9538512]
46. Alattia JR, Shaw JE, Yip CM, Privé GG. Direct Visualization of Saposin Remodelling of Lipid Bilayers. *J Mol Biol*. 2006; 362:943–953. DOI: 10.1016/j.jmb.2006.08.009 [PubMed: 16949605]
47. Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. *J Mol Graph*. 1996; 14:33–8–27–8. [PubMed: 8744570]

Highlights

- Bacterial expression of the saposin-like sub-domain of human Wnt3a was achieved when the first 82 N-terminal residues of Wnt3a were included.
- Far UV circular dichroism spectroscopy of N terminal saposin-like sub-domain (NT-SLD) of Wnt3a yielded high α -helix secondary structure at neutral pH.
- Unlike prototypical saposins, NT-SLD of Wnt3a exhibited weak membrane-binding affinity and lacked cell lytic activity.
- NT-SLD was unable to induce stabilization of β -catenin or modulate the extent of β -catenin stabilization induced by full-length Wnt3a.

Figure 1. Structural models of *Xenopus* Wnt8 and NT-SLD

Panel A) Structure of *Xenopus* Wnt8 complexed with Fzd8-CRD (Protein Data Bank entry: 4FOA) was generated using Visual Molecular Dynamics [47]. The first NT 82 amino acids are in magenta, the SLD is multicolour and the CT domain is in grey. Fzd8-CRD is shown as grey space-fill. The position of 4 tryptophan residues are marked with stars, the remaining tryptophans in the SLD portion of Wnt8 reside in a region masked by the presence of Fzd8-CRD. Panel B) Schematic representation of secondary structure elements of human Wnt3a NT-SLD (adapted from [43]). Cylinders depict α -helices; block arrows indicate β -sheet and dotted lines connect cysteine disulfide bonds. Individual α -helices are color-coded: magenta (N terminal 82 amino acids), cyan (SLD helix a), green (helix b), yellow (helix c) and red (helix d). 'W' followed by their sequence position indicates the location of SLD tryptophan residues.

Figure 2. SDS-PAGE of isolated SLD proteins

Lane assignments: lane 1) prestained Precision Plus protein standard, lane 2) NT-SLD (expected molecular weight = 25 kDa) and lane 3) NT-SLD(nh) (expected molecular weight = 20 kDa). Five μ g protein was applied to each well and, following electrophoresis, the gel was stained with Gel Code Blue.

Figure 3. Far UV CD spectroscopy of SLDs

Panel A) Spectra of NT-SLD (0.2 mg/ml) in 50 mM Na citrate pH 3, 20 mM NaCl (solid line), 50 mM Na citrate, pH 3, 20 mM NaCl plus 1% SDS (dotted line) or 50 mM sodium citrate, pH 3, 20 mM NaCl plus 50% trifluoroethanol (TFE) (dashed line). Panel B) NT-SLD (solid line) and NT-SLD(nh) (dashed line) in 20 mM NaPO₄, pH 7.2, 2% DTAC.

Figure 4. SLD-liposome binding interactions

Liposomes were incubated in the presence or absence of NT-SLD or NT-SLD(nh) at a liposome:protein ratio = 20:1 (w/w). After 1 h incubation, the samples were subjected to sucrose gradient centrifugation. Following centrifugation, five fractions (200 μ l each) were collected from top to bottom (1 to 5). The liposome content of each fraction was detected by measuring light scattering intensity at 310 nm and presented graphically (Panel A). An aliquot of each fraction was analyzed by SDS-PAGE and proteins visualized by Imperial protein stain (Panel B). Results presented are representative of two independent experiments.

Figure 5. Fluorescence emission spectra of SLDs

Left) Tryptophan fluorescence emission spectra (excitation 280 nm) of (a) NT-SLD (1 mg/ml) in 10 mM Na phosphate, pH 7, 0.6 M arginine HCl, recorded from 300 to 450 nm in the absence (solid black line) and presence (dotted black line) of 2% DTAC. Right) NT-SLD(nh) (1 mg/ml) in 10 mM Na phosphate, pH 7, 0.6 M arginine HCl, recorded from 300 to 450 nm in the absence (solid blue line) and presence of 2% DTAC (dotted blue line). Fluorescence spectra for NT-SLD and NT-SLD(nh) in 2% DTAC were normalized against respective control samples.

Figure 6. Thrombin-mediated proteolysis of NT-SLDs

NT-SLD and NT-SLD(nh) were incubated in the absence and presence of thrombin in 10 mM Na phosphate, pH 7, 0.6 M arginine HCl, labeled as buffer (panel A) and buffer plus 1% CHAPS (panel B) in final volume of 10 μ l for 1, 2 and 4 h at 37°C. Control SLD incubations in the absence of thrombin were incubated for 4 h at 37°C. Following incubation, samples were subjected to SDS-PAGE and stained with Imperial protein stain. The results are representative of two experiments.

Figure 7. Effect of SLDs on L cell viability

Cultured murine L cells were incubated in the absence and presence of increasing concentrations of NT-SLD or NT-SLD(nh) for 24 h. Following incubation, cell viability was measured by the MTT assay. SLDs were presented to the cells in 10 mM Na phosphate, pH 7, 0.6 M arginine HCl. Values reported are the mean $\pm$ standard error (n=3).

Figure 8. Effect of SLD on canonical Wnt signaling activity

L cells were incubated with indicated concentrations of Wnt3a and (A) NT-SLD or (B) NT-SLD(nh) for 3 h. Following incubation, the cells were lysed and 10 μ g lysate protein applied to a 10% SDS-PAGE gel. Separated proteins were transferred to a PVDF membrane and probed with antibodies directed against β -catenin and GAPDH. The results are representative of three independent experiments.