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

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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 50–60% 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.

1. 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, ubiquitinylated and degraded by proteasomes [4]. Spatio-temporal 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

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.

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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 mono-unsaturated 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.

2. Materials and methods

2.1. 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. Pre-stained 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 Advansta.

2.2. 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′ - ctccaccacctc-gagcatctccga - 3′). The same human *WNT3A* template was used to amplify a 666 bp sequence (NT-SLD) using FwdNTSLDNde1 (5′-atcatatgagctacccgatctggtggtcg - 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].

2.3. Recombinant protein production and purification

Bacteria were cultured in NZCYM media plus ampicillin (50 μg/ml) at 30 °C. When the OD_{600} 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 20,000 g 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 20,000 g 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 20,000 g for 15 min. The supernatant was filtered (0.22 μm) 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 H_2O, lyophilized and stored at −20 °C.

2.4. Recombinant protein solubilization

Lyophilized protein was re-suspended (4 mg/ml) in 2% DTAC (w/v) in the presence of 2 μM oxidized glutathione and 10 μM 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)