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Structural Studies of Human Naked2: A Biologically Active Intrinsically Unstructured Protein

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Abstract

Naked1 and 2 are two mammalian orthologs of Naked Cuticle, a canonical Wnt signaling antagonist in *Drosophila*. Naked2, but not Naked1, interacts with transforming growth factor- α (TGF α) and escorts TGF α -containing vesicles to the basolateral membrane of polarized epithelial cells. Full-length Naked2 is poorly soluble. Since most functional domains, including the Dishevelled binding region, EF-hand, vesicle recognition and membrane targeting motifs, reside in the N-terminal half of the protein, we expressed and purified the first 217 residues of human Naked2 and performed a functional analysis of this fragment. Its circular dichroism (CD) and nuclear magnetic resonance (NMR) spectra showed no evidence of secondary and/or tertiary structure. The fragment did not bind calcium or zinc. These results indicate that the N-terminal half of Naked2 behaves as an intrinsically unstructured protein.

Keywords

Naked2; intrinsically unstructured protein; circular dichroism; nuclear magnetic resonance; transforming growth factor- α

Abbreviations

IUP, intrinsically unstructured protein; CD, circular dichroism; GST, glutathione S-transferase; NMR, nuclear magnetic resonance; IPTG, Isopropyl-beta-D-thiogalactopyranoside; TGF α , transforming growth factor- α

Introduction

Canonical Wnt signaling has emerged as a critical regulator of both stem cells and cancer cells [1]. Naked1 and Naked2 are two mammalian orthologs of *Drosophila* Naked Cuticle, which has been shown to negatively regulate canonical Wnt signaling through an interaction with Dishevelled [2–5]. Both Naked1 and Naked2 have been proposed to interact with Dishevelled through their EF-hand-like motif (residues 137–157) [3,6]. In addition, Naked2 has been reported to bind to Dishevelled through its TGF α binding region [7]. Naked1 has been shown to be highly upregulated in human colorectal cancers [8].

Naked Cuticle and its mammalian homologues have different distributions. Naked Cuticle is localized in both the cytoplasm and nucleus of *Drosophila* cells [Waldrop et. al. *Genetics*, In

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press]. In contrast to Naked Cuticle, both Naked1 and 2 contain an N-myristoylation site and localize predominantly to vesicles and the plasma membrane [7, Li et. al. J. Cell Biol., Submitted].

Naked2, but not Naked1, interacts with the cytoplasmic C-terminal fragment of a Golgi-processed form of TGF α , coats TGF α -containing exocytic vesicles and escorts those vesicles to the basolateral membrane of polarized epithelial cells [7]. Myristoylation of the N-terminal glycine residue of Naked2 is required for fusion of Naked2-associated vesicles to the plasma membrane. The first 36 residues of Naked2 contain an exocytic vesicle recognition motif and the residues 1–173 redirect Na⁺/H⁺ exchange regulatory factor-1 from the apical cytoplasm to the plasma membrane of polarized MDCK cells. Thus, information for basolateral directionality is encoded within Naked2 residues 37–173, which include the EF-hand region [Li et. al. J. Cell Biol., Submitted].

Recently, PR72 and PR130, two protein phosphatase type 2A regulatory subunits, have been found to form a complex with Naked1 and 2 and regulate Wnt signaling through activation or repression of Naked, respectively; however, the significance of this regulation is unknown [9,10]. Naked1 mutant mice with a targeted disruption of the EF-hand region show no observable phenotype except impaired spermatogenesis [11]. This result suggests that either the EF-hand region might not be a critical domain for many of the functions of Naked1, or that Naked2 is functionally redundant with Naked1.

All the above observations indicate that Naked1 and 2 are biologically active proteins. Naked2 binds to multiple proteins and may function as a switch protein through its several functional motifs. Structural information may provide important insights into the function and regulation of Naked2. Efforts to express either full-length Naked2 or its 1–331 fragment resulted in insoluble proteins. Structural prediction algorithms DISOPRED2 and DisEMBL [12,13] indicated that the C-terminus of Naked2 is highly disordered; however, these algorithms suggested possible secondary structure in the N-terminus of the protein. Combined with the fact that most of the Naked2 functional domains, including myristoylation, EF-hand motif, Dishevelled binding region, vesicle recognition and membrane targeting motif are in the N-terminal region of Naked2 [4,5,7], we decided to test those predictions experimentally. We expressed and purified residues 1 through 217 of Naked2 (Naked2_{1–217}) including the EF-hand-like motif. The structures were analyzed using CD and NMR spectroscopy.

Materials and Methods

Materials

The pGEX-2TK vector was from Amersham (Piscataway, NJ). *E. coli* BL-21(DE3)pLysS competent cells were from Stratagene (La Jolla, CA). Protease inhibitor cocktails, benzamidine and lysozyme were from Sigma (St. Louis, MO). Immobilized glutathione agarose was from Pierce (Rockford, IL). Thrombin cleavage kit was from Novagen (Madison, WI). Biosafe Coomassie blue staining kit was from BioRad (Hercules, CA). Amicon Ultra centrifugal filters were from Millipore (Bedford, MA). Isopropyl-beta-D-thiogalactopyranoside (IPTG) was from RPI (Mt. Prospect, IL).

Expression and purification of human Naked2_{1–217}.

The target Naked2_{1–217} gene was PCR-amplified from full-length Naked2 cDNA. The fragment was ligated into BamHI and EcoRI sites of a bacterial expression vector pGEX-2TK. The construct was transformed into *E. coli* strain BL21(DE3)pLysS competent cells and plated onto LB plates in the presence of ampicillin. A single colony was picked and grown overnight at 230 rpm, 37°C in 30 ml LB media containing 100 µg/ml ampicillin and then transferred to

1 L LB media and continuously shaken at 37 °C until OD₆₀₀ reached 0.7. The expression of the Naked2₁₋₂₁₇ fragment was induced by adding 0.2 mM IPTG for 18 hrs at 230 rpm, 24 °C. Cells were harvested by centrifugation for 20 min at 4000 rpm in a Sorvall SLA-1500 rotor and the pellet was re-suspended in 30 ml lysis buffer (50 mM Tris-HCL pH 8.0, 300 mM NaCl, 1 mM EDTA, 100 μM benzamidine, lysozyme and protease inhibitor cocktail). After 30 min incubation on ice, the lysate was sonicated three times for 20 sec and then spun at 13,000 g for 30 min. The supernatant was applied to 3 ml glutathione-agarose beads and incubated at room temperature for 30 min. After four washes with 30 ml lysis buffer, agarose beads were incubated with 4 units biotinylated thrombin in 10 ml lysis buffer for 16 h at room temperature. The supernatant was then incubated with 50 μl of streptavidin-agarose beads for 30 min to absorb the thrombin. Protein concentration was determined by the Bradford method. The purity of the protein was determined by SDS-PAGE, Coomassie blue staining and western blotting.

Western blotting

The protein sample was resolved by 10% SDS-PAGE and transferred to Nitrocellulose membranes (Waterman). The blot was then blocked with 5% milk and incubated with a rabbit polyclonal antibody against residues 44–65 of human Naked2 (1:1000 dilution) followed by incubation with horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were detected using enhanced chemiluminescence.

CD spectroscopy

CD spectra were recorded on a Jasco J-810 spectropolarimeter using a 1 mm path-length quartz cuvette at room temperature. Naked2₁₋₂₁₇ was adjusted to a final concentration of 10 μM in PBS. The spectrum was obtained by averaging four successive individual scans over the 195–260 nm wavelength range. The respective buffer solvent backgrounds were subtracted. The α -helix content (%) was calculated using the software CDNN [14].

NMR experiments

Experiments were carried out on a natural abundance 0.25 mM Naked2₁₋₂₁₇, (0.3 M NaCl, 50 mM Tris, pH 7.5). Metal ions were added from 1 M stock solutions of CaCl₂ and ZnCl₂. The sample Ca²⁺ and Zn²⁺ ion concentrations were increased between 0.5 and 2.0 mM, corresponding to final 8-fold molar excess over protein. A number of ¹H one-dimensional (1D) and two-dimensional (2D) NMR experiments were recorded on an Avance500 (11.8 T) spectrometer equipped with a cryoprobe (Bruker). The frequency sweep width was adjusted to 6009 Hz and 2048 complex points of the free induction decay were averaged over 128 and 32 scans in 1D and 2D experiments, respectively. The carrier frequency was set on the H₂O signal. One D experiments were done at 19 °C. NOESY experiments [15] were recorded at 19, 29 and 39 °C with time proportional phase incrementation [16] used for 600 t₁ values and 100 ms mixing time. Pulsed field gradient water suppression was used in all experiments [17].

Results

The purification of human Naked2₁₋₂₁₇

We constructed the GST-Naked2₁₋₂₁₇ to allow affinity purification on glutathione-agarose beads. Protein purification and GST removal were performed on the beads. Thrombin digestion of agarose-bound GST-Naked2₁₋₂₁₇ released free Naked2₁₋₂₁₇. The purified protein is shown in Fig. 1A. The GST-Naked2₁₋₂₁₇ was expressed abundantly after IPTG induction and remained mostly in the soluble fraction. A single ~26 kD band was detected in SDS-PAGE after thrombin digestion and the identity of this band was confirmed to be Naked2₁₋₂₁₇ by antibody detection (Fig. 1B).

CD measurements

To identify secondary structural elements, we recorded CD spectra of the Naked₂₁₋₂₁₇. The spectrum showed no features characteristic of either α -helix or β -strand conformations. The possible α -helix content was calculated to be less than 6% by CDNN software [14]. These results suggested that the 1–217 fragment is mostly unstructured in solution.

NMR Studies

The solution 1D ¹H NMR experiments with Naked₂₁₋₂₁₇ in a pH range between 5.5 and 7.5 showed marginal dispersion of resonance signals typical for disordered polypeptides (Figure 3A). Addition of Ca²⁺ or Zn²⁺ did not change the dispersion in 1D and 2D spectra under the experimental conditions tested in our studies. NOESY experiments did not indicate stable tertiary interactions between backbone amide hydrogens of Naked₂₁₋₂₁₇ with and without divalent metal ions present. The distribution of amide signals characteristic of unstructured polypeptides (centered at approx. 8.2 ppm) and their cross-peak signals did not change significantly with temperature between 19 and 39 °C. The areas of weak and broadened cross peaks observable in NOESY spectra most likely correspond to intraresidue and sequential interactions in a region of nascent structure (see Discussion).

Discussion

Intrinsically unstructured proteins (IUPs) are common in metazoan proteomes and occupy a unique niche in which function does not rely on stably formed tertiary structure. The significance of protein disorder is emphasized by its association with a number of cellular processes: signal transduction, cell-cycle regulation, gene expression and chaperone action [12,18–20]. Naked₂ is involved in both Wnt signaling and TGF α trafficking [2,7]. Structure prediction algorithms suggest very little secondary and/or tertiary structure for Naked₂ [12, 13]. Hence we tested the hypothesis that Naked proteins are biologically active IUPs. Full-length Naked₂ was insoluble under all conditions employed and so were C-terminally deleted forms of Naked₂. Full-length Naked₂ or even constructs containing residues 1–331 are insoluble and therefore not amenable to solution studies. The 217 N-terminal residues constitute the largest soluble fragment of human Naked₂ and it contains the conserved EF-hand motif, thought to be a key functional element.

The N-terminal half of Naked₂, which contains the EF-hand-like region, is stable in solution. We cloned, expressed and purified Naked₂₁₋₂₁₇ fragment using GST-affinity purification method. The protein appeared to be greater than 90% pure by SDS-PAGE and Coomassie blue staining (Fig. 1A) and was identified as a single band on western blotting using a Naked₂ specific antibody (Fig 1B).

However, both CD and NMR measurements indicate that isolated human Naked₂₁₋₂₁₇ is mostly unstructured in solution. This polypeptide was soluble and covalently stable in conditions corresponding to physiological ionic strength and pH. In NOESY experiments, we detected no tertiary structure (Figure 3B, lower half). A possible segment of nascent secondary structure indicated by areas of broad cross peaks in NOESY experiments (Figure 3B, upper half) and observed weak helicity in CD measurements could be localized to residues 137–157 corresponding to the putative EF-hand motif. Several protein disorder prediction algorithms [12, 13] also suggest mostly unstructured state for the entire length of Naked₂, with the possible exception of the EF-hand motif. This region of Naked Cuticle was initially postulated to bind calcium [5] but this was questioned by a subsequent study showing that it was unlikely to bind to Ca²⁺ [6]. Our NMR experiments showed no observable changes upon addition of Ca²⁺ to human Naked₂₁₋₂₁₇. Additionally, Naked₂₁₋₂₁₇ showed no apparent binding of Zn²⁺, although such binding was reported for full-length Naked₂ [6]. Calcium and zinc binding motifs may

exist in the remaining 234 residue long C-terminal portion of the protein or they may be formed when Naked2 interacts with its cognate binding partners *in vivo*.

Human Naked1 and 2 are only 40% identical and they are ~70% identical to their orthologs in mouse. The intrinsically unstructured nature of these proteins can accommodate more sequence variation despite their participation in very conserved cellular processes [21]. *Drosophila* Naked Cuticle (928 residues) and human Naked2 (451 residues) show similarity only between residues 105–188 of the human and 171–258 of the *Drosophila* sequence. This more highly conserved region including the EF-hand motif has 35 identities over 88 residues. For IUPs, there can be little selective pressure on conservation of structurally critical elements, as long as human and *Drosophila* Naked share minimal sequences required for their functions. Although *Drosophila* Naked Cuticle and human Naked2 vary greatly in sequence, they might form similar conformations when bound to their cognate partners.

Several well-documented examples show at least parts of IUPs assuming stable structure when bound to their partners. For example, cyclin kinase inhibitor p27 is an IUP but becomes largely structured when bound to Cdk/cyclin targets [22]. Naked2 has been shown to bind to Dishevelled, TGF α , PR72 and PR130 [3,7,9,10]. It is possible that Naked2 might undergo conformational change or form a stable structure when bound to these interacting proteins.

In conclusion, results presented here support the hypothesis that Naked2 is an IUP. Its EF-hand like motif does not directly bind to either calcium or zinc ions. Naked2 binds to several proteins *in vivo* and its bound conformation and metal ion binding may be defined by the components of the active complex.

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Fig. 1. SDS-PAGE analysis of the Naked2₁₋₂₁₇ expression and purification

Human Naked2₁₋₂₁₇ fragment was expressed and purified as described in “Materials and Methods”. Samples from different stages were collected and analyzed with SDS-PAGE and Coomassie blue staining. Fig 1A, Lane 1: molecular mass markers; 2: *E. coli* total cell proteins before induction by IPTG; 3: *E. coli* total cell proteins 16 h after induction with IPTG; 4: Total cell lysate after lysozyme treatment and sonication; 5: Supernatant after centrifugation; 6: Elution after thrombin cleavage on agarose beads for 16 h. Fig 1B, the eluate (Fig 1A lane 6) was analysed by western blotting using a rabbit polyclonal antibody against residues 44–65 of human Naked2.

Fig.2. CD spectrum of the Naked2₁₋₂₁₇

Naked2₁₋₂₁₇ was adjusted to 10 μ M in PBS. The spectrum was obtained by averaging four successive individual scans over the 195–260 nm wavelength range.

Fig.3. NMR spectra of Naked2₁₋₂₁₇

(A) Amide and aliphatic portions of ¹H 1D spectra of 0.25 mM Naked2₁₋₂₁₇, Tris-HCl 50 mM, NaCl 300 mM, pH 7.5, 19 ° C. Spectra with added Ca²⁺ and Zn²⁺ are labeled. The region between 3 and 6 ppm (not shown) was obscured by intense solvent and buffer signals. (B) Amide portions of 2D ¹H NOESY experiments recorded at temperatures indicated. Sample was 0.25 mM Naked2₁₋₂₁₇, Tris-HCl 50 mM, NaCl 300 mM, 2 mM CaCl₂, pH 7.5. The horizontal lines at 3.7 and 4.7 ppm are artifacts caused by buffer and water signals, respectively.