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The C-terminus of ICln is Natively Disordered but Displays Local Structural Preformation

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Key Words
ICln • Structure • NMR • Intrinsic disorder

Abstract
ICln is a vital, ubiquitously expressed protein with roles in cell volume regulation, angiogenesis, cell morphology, activation of platelets and RNA processing. In previous work we have determined the 3D structure of the N-terminus of ICln (residues 1-159), which folds into a PH-like domain followed by an unstructured region (residues H134 – Q159) containing protein-protein interaction sites. Here we present sequence-specific resonance assignments of the C-terminus (residues Q159 – H235) of ICln by NMR, and show that this region of the protein is intrinsically unstructured. By applying $^{13}C\alpha$-$^{13}C\beta$ secondary chemical shifts to detect possible preferences for secondary structure elements we show that the C-terminus of ICln adopts a preferred α-helical organization between residues E170 and E187, and exists preferentially in extended conformations (β-strands) between residues D161 to Y168 and E217 to T223.

Introduction

ICln is an ubiquitous protein which partakes in essential physiological pathways regulating cell volume [1-2], cell morphology [3], angiogenesis [4], the activation of platelets [5-6], and RNA processing [7-9]. In line with the diverse roles of ICln in these vital physiological pathways, knock-out of ICln is lethal [10], and altered expression levels of ICln may contribute to diseases like spinal muscular atrophy [11].

As a multifunctional protein and potential connector hub ICln interacts with a variety of partner proteins in different compartments in the cell. ICln for example interacts with components of the cytoskeleton like actin [12-14] or the protein 4.1 [15-16], while interactions of ICln with RNA processing factors like LSm and Sm proteins or the protein arginine methyltransferase PRMT5 most likely take place in the cytosol [7-9, 17-19]. Furthermore, a fraction of ICln is located at the plasma membrane [6, 14, 20-22], where it is functionally associated with the swelling dependent anion channel [1-2, 23-24] and interacts with HSPC038 [25] or the integrin $\alpha_{IIb}\beta_3$ [5-6].

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Given the diverse roles of ICln as well as the number of interaction partners described so far (reviewed in [26]), knowledge of the 3D structure of ICln is highly desirable to pin down potential binding sites of partner proteins and thus the structure-function relation of this essential protein.

The instability of purified ICln in solution prevented the structure determination of full length ICln by NMR, and, so far, a 3D structure of the N-terminus of ICln, i.e. residues 1-159 of canine ICln (ICln(1-159)), has been solved [19, 27]. This region of ICln folds into a PH-like domain which is followed by an unstructured region located between amino acids (AA) H134 and Q159 [19]. The hallmarks of the PH-like domain of ICln are its overall negative surface potential and an unusually long, unstructured loop connecting β-strands 6 and 7, which set it apart from many of the other PH-domains described so far [19]. The exact function of the PH-like domain of ICln is not fully understood but may involve the plasma membrane, as this region of the protein contains a binding site for the zinc finger protein HSPC038 [25] and a potential binding site for integrin $\alpha_{IIb}\beta_3$ [5], ICln interaction partners involved in swelling dependent anion channel (IClswell) [25] or platelet [5] activation, respectively. Interaction sites of ICln partner proteins are also located in the unstructured region of ICln between AA H134 and Q159 [19, 25], or in the region between Q159 and H235 [3, 18] (reviewed in [26, 28]), for which no structure is available so far. To get further insight into the structure-function relation of ICln, we therefore set out to determine the structure of the C-terminus of ICln by NMR, i.e. the region between residues Q159 and H235 of canine ICln.

Materials and Methods

Protein cloning and expression

The ORF of full length canine ICln (accession number X65450) as well as a DNA fragment corresponding to residues Q159-H235 of canine ICln were amplified by PCR and cloned into the bacterial expression vector pET3-His [29] (Xho I and Bam HI restriction sites) using standard methods [19, 25]. When expressed in *E. coli* the resulting proteins contain an N-terminal His_6-tag (MHHHHHHLE) in frame with residues 1 to 235 (ICln(1-235)) or Q159 to H235 (ICln(159-235)) of canine ICln. The final ICln constructs thus comprise 244 (ICln(1-235)) and 86 (ICln(159-235)) residues, respectively. For protein expression and uniform labeling with ^{15}N and ^{13}C, transformed *E. coli* BL21(DE3) Gold (Agilent Technologies, Austria) were first grown in ZB-medium (0.5 % (w/v) NaCl and 1 % (w/v) of NZ-amine AS (Sigma, Austria)) and then diluted 1:100 into 1 liter of minimal medium (48 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.56 mM NaCl and 18.7 mM $^{15}NH_4Cl$ (Euriso-top, Germany), supplemented with 20 ml of an 18 % (w/v) [^{13}C]-D-Glucose (Euriso-top, Germany) solution, 2 ml of 1 M $MgSO_4$, 4 ml of 10 mM $ZnSO_4$, 0.1 ml of 1 M $CaCl_2$ and 100 µg/ml Ampicillin) and further cultured at 37 °C in an orbital shaker (Sanyo, Austria). Protein expression was induced at an OD_{600} of ~0.8 by addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.5 mM. Bacterial cells were harvested 6.5 h after induction.

Protein Purification

The bacterial pellet obtained from a 1 liter culture in minimal medium was resuspended in 30 ml of lysis buffer (25 mM K_2HPO_4, 150 mM NaCl, 10 mM imidazole, 5% glycerol, pH 7.2) and cells disrupted using a French press (SLM Aminco, USA). The bacterial lysate was cleared by centrifugation and loaded onto an IMAC column (HisTrap, GE Healthcare, Austria) pre-equilibrated with lysis buffer. Following extensive washing with lysis buffer, bound protein was eluted with a buffer containing 25 mM K_2HPO_4, 150 mM NaCl, 200 mM imidazole, 5 % glycerol, pH 7.2. Fractions containing ICln(159-235) were pooled, concentrated (Amicon Ultra 5 kDa ultrafiltration units (Millipore, Austria)) and loaded onto a Sephacryl S-100 HR column (GE Healthcare, Austria) equilibrated in 25 mM K_2HPO_4, 150 mM NaCl, pH 7.2. Fractions containing ICln(159-235) were collected, pooled, concentrated as described above and stored at -70 °C until use.

For the purification of N-terminally His_6-tagged full length ICln (ICln(1-235)) IMAC was followed by anion exchange chromatography (Source Q, GE Healthcare, Austria; wash buffer: 25 mM K_2HPO_4, 150 mM NaCl, 5 % glycerol, pH 7.2; elution buffer: 25 mM K_2HPO_4, 2 M NaCl, 5 % glycerol, pH 7.2) prior to the final gel filtration step.

Nuclear Magnetic Resonance (NMR) spectroscopy

NMR spectra were obtained at 25 °C on Varian Inova 800 MHz and 500 MHz spectrometers. The experiments performed included ^1H-^{15}N HSQC, 3D HNCO, 3D HNCACO, 3D HNCACB, and 3D CBCA(CO)NH [30]. NMR data were processed and analyzed with the NMRPipe [31] and NMRView [32] software packages. The amino acid assignments have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) under BMRB accession number 5736.

Results

To determine the structure of the C-terminus of ICln, a truncation mutant comprised of amino acids (AA) Q159 to H235 of canine ICln (ICln(159-235)) was cloned as fusion protein with a His_6-tag, over-expressed in *E. coli*, purified (Fig. 1, inset; yield: ~4 mg/l expression culture) and subjected to NMR spectroscopy for amino acid assignment and structure determination.

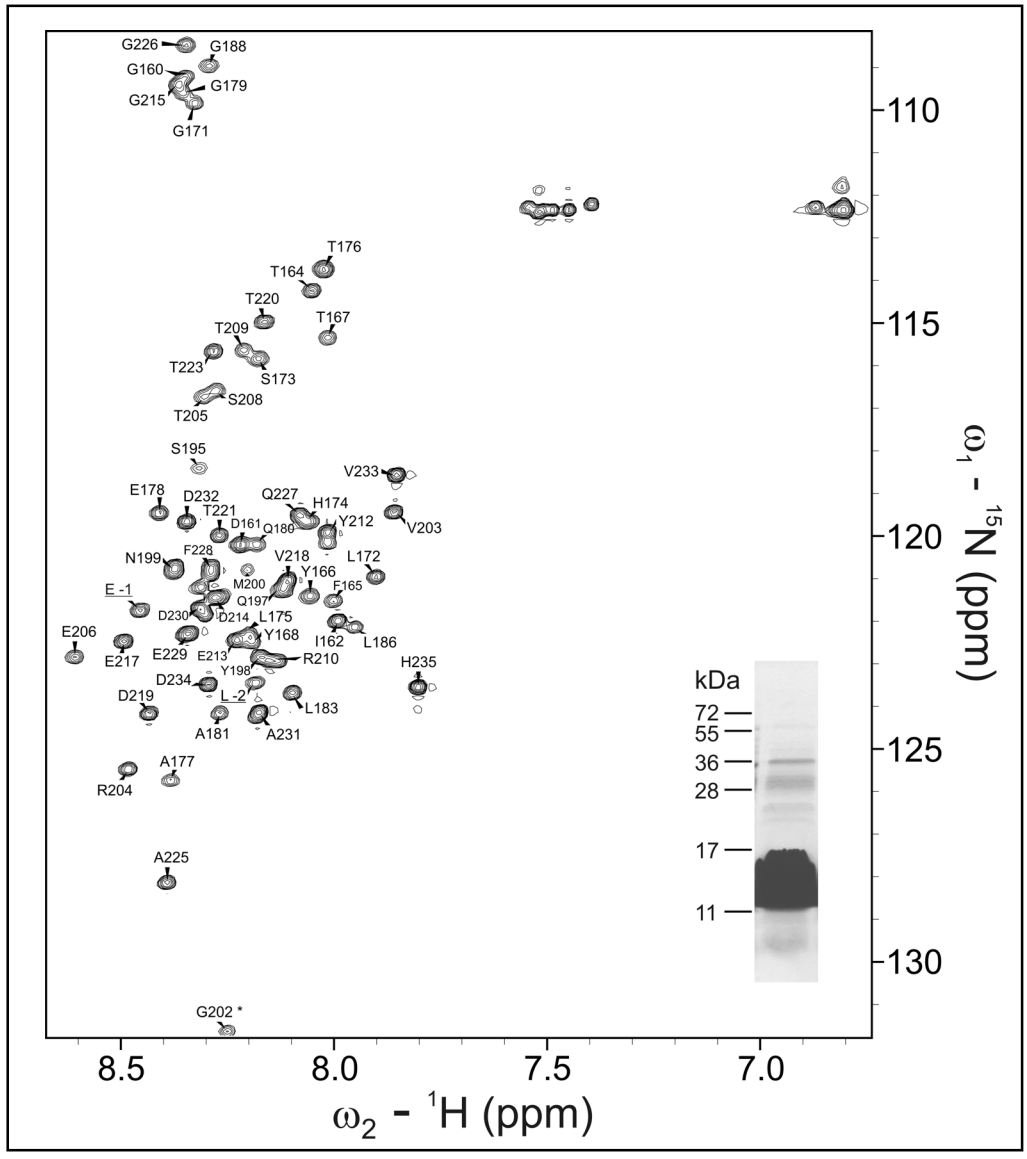

Fig. 1. ^{1}H-^{15}N HSQC spectrum of ICln(159-235) and amino acid assignment. Amino acids "E -1" and "L -2" are part of the His_6-tag (-9 MHHHHHH**LE** -1; numbering refers to the start of ICln specific sequence) fused to ICln(159-235). Note that G202* whose real ^{15}N chemical shift I_{real} is outside of the selected ^{15}N spectral width SW appears at the aliased ^{15}N frequence position I_{obs} ($I_{obs}=I_{real}+SW$) in the spectrum. The inset shows a Coomassie stained SDS-PAGE overloaded with purified ICln(159-235).

High-quality NMR data for ICln(159-235) was obtained as shown by the ^{1}H-^{15}N HSQC spectrum collected at 800 MHz (Fig. 1). In total 61 of the 75 non-proline ^{1}H and ^{15}N backbone resonances of ICln(159-235) (starting methionine, his-tag omitted) were assigned (81.3 %) (Fig. 1).$^{13}C\alpha$, $^{13}C\beta$ and ^{13}CO chemical shifts were allocated for 71 residues (92.2%). Impossibility of complete backbone assignment was due to missing resonances and signal overlap. The experimentally derived secondary structure (Fig. 2A) was compared with predicted secondary structure elements obtained by applying the meta-structure approach [33] (Fig. 2B). The meta-structure was recently introduced as a novel tool for protein sequence analysis and provides quantitative parameters (compactness and secondary structure) about protein topology. The meta-structure derived secondary structure parameter is defined in analogy to well-established $^{13}C\alpha$ secondary shifts, with positive values for an α-helix and negative values indicating the presence of an extended conformation. Details of the calculation procedure and its usefulness for studies of intrinsically unstructured proteins (IUPs) have already been described [33-34]. Figure 2 shows a comparison with experimental $^{13}C\alpha$-$^{13}C\beta$ secondary shifts. Overall, the location of extended conformations and helices are correctly identified and there is very good agreement between prediction and experiment. Additionally, the distribution of compactness values in the C-terminus (Fig. 2B) suggests the existence of compact structural segments in ICln(159-235). A similar observation was made in the case of Osteopontin, an intrinsically disordered cytokine involved in metastasis and tumor progression [34]. We thus conclude that although being largely disordered/unstructured the C-terminus of

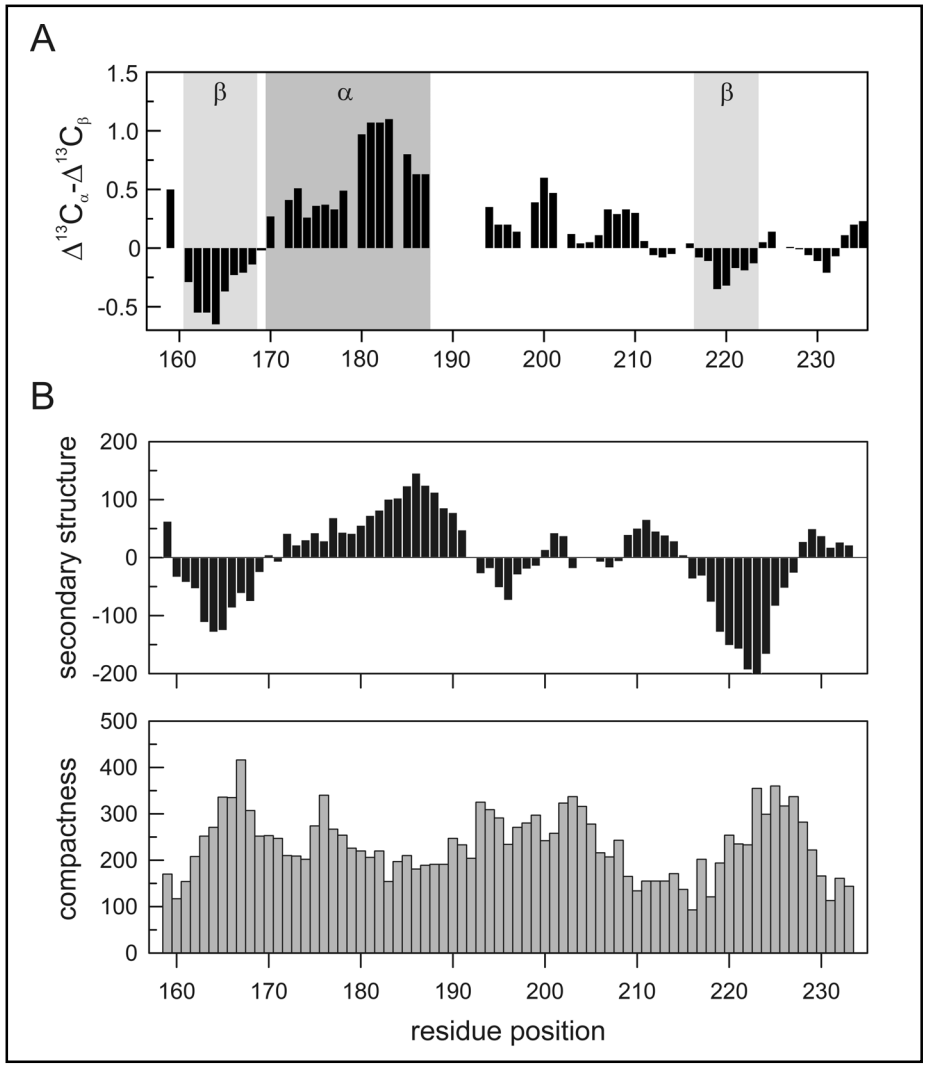

Fig. 2. (A) Secondary structure of ICln(159-235) probed by NMR spectroscopy. (B) Meta-structure analysis of ICln(159-235). The meta-structural parameters secondary structure (black bars) and compactness (grey bars) are shown. (top) α-helical regions display positive values, whereas negative values are observed for extended or β-strand regions. (bottom) Large compactness values are found for residue positions located in compact regions of the protein structure, while small values are found for conformationally flexible residues exposed to the solvent.

ICln transiently populates partially folded structures (α-helical: AA E170 to E187; extended (β-strands): AA D161 to Y168 and AA E217 to T223) which are in dynamic exchange with less compact conformations, ranging from extended coils to more compact structures exhibiting distinct electrostatic interactions. These transiently (pre-) formed structures and the concomitant structural plasticity are likely to be of relevance for the interaction with diverse binding partners.

ICln(159-235) which has a calculated MW of 9.69 kDa and a theoretical pI of 4.47 shows a lower relative electrophoretic mobility during SDS-PAGE (as estimated by comparison with molecular weight markers using ImageJ) corresponding to an apparent MW of 14.05 ± 0.83 (mean ± stdev; n=5). A similar observation of lower electrophoretic mobility in SDS-PAGE was made for the DNA repair protein XPA [35] or the alpha s1-casein [36] and the altered electrophoretic mobility explained by a high acidic AA content and intrinsic disorder or extended structures in the protein.

To verify if the last 77 amino acids of canine ICln are also natively unstructured in the full length ICln(1-235) protein, NMR experiments with ^{15}N-labeled His_6-tagged ICln(1-235) were performed. The overlay of the spectra obtained from ICln(159-235) and of full length ICln(1-235) (Fig. 3) shows that there were no evident differences between the signals of the C-terminal 77 residues in full length ICln(1-235) and the truncation mutant ICln(159-235). This finding indicates that the N-terminal His_6-tag has no apparent influence on structure formation in either His_6-tagged ICln(1-235) or ICln(159-235) and demonstrates that also in full length ICln the residues Q159 to H235 lack a well-defined three-dimensional structure and show the same secondary structure propensity as determined for the truncation mutant ICln(159-235).

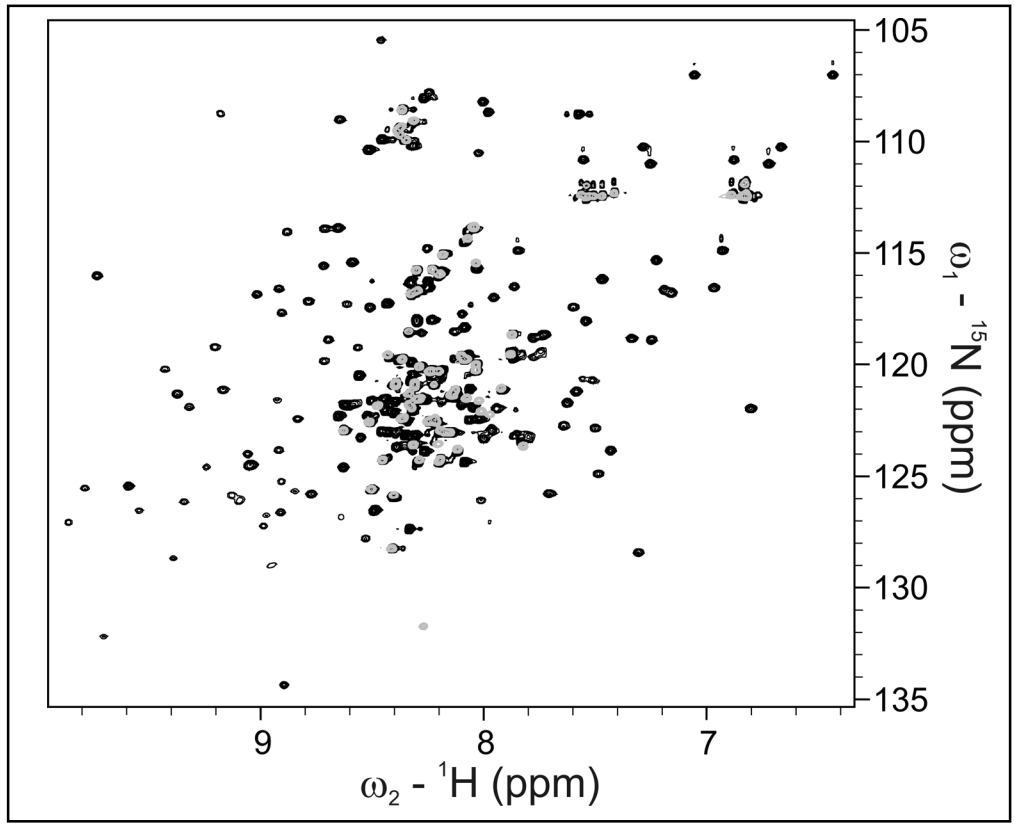

Fig. 3. Overlay of the NMR spectra of full length ICln(1-235) (black) and the truncation mutant ICln(159-235) (grey).

Discussion

In the eukaryotic proteomes intrinsically unstructured proteins (IUPs) are common and can be either completely unstructured or - similar to ICln - partially folded [37-38]. Natively unstructured regions in a protein have some features in common, for example a low number of hydrophobic residues, a high net charge, as well as enrichment in particular polar and charged amino acids, and frequently serve as binding sites for partner proteins (reviewed in [39-41]).

In ICln three regions rich in negatively charged amino acids have been proposed as protein-binding sites [12, 18]. In canine ICln the acidic region (AR) 1 is located between AA E96 and E105, and is part of an unusually long unstructured loop connecting beta strand 6 and 7 of the ICln PH-like domain [19]. As shown in [19] and this work, also AR 2 (AA D136 - E153) and AR 3 (AA E229 - H235) are located in the unstructured regions of the ICln protein. NMR and mutagenesis experiments demonstrated that AR2 is part of the binding site for HSPC038 [25] and AR3 for the protein arginine methyltransferase PRMT5 [18], which may suggest that the unstructured C-terminus of ICln serves as a protein-protein interaction domain.

	UniProt Acc. Nr.	Homology (%)	IUPred score
Cf ICln(134-235)	P35521	100	0.73 ± 0.12
Hs ICln(136-237)	P54105	99	0.71 ± 0.12
Oc ICln(135-236)	Q28678	99	0.71 ± 0.12
Mm ICln(135-236)	Q61189	97	0.73 ± 0.12
Xl ICln(139-241)	P54106	77	0.80 ± 0.10
Dr ICln(142-249)	P79725	73	0.77 ± 0.13

Table 1 Sequence homology and disorder prediction of the C-terminus of ICln. For analysis, the entire region C-terminal to the ICln PH-like domain (AA H134-H235 of canine ICln) was chosen and aligned to the respective region of ICln of other vertebrates using LAlign [49]. Numbers in brackets denote the region in the protein sequence of canine (*Canis familiaris* (Cf)), human (*Homo sapiens* (Hs)), rabbit (*Oryctolagus cuniculus* (Oc)), mouse (*Mus musculus* (Mm)), amphibian (*Xenopus laevis* (Xl)) and teleost (*Danio rerio* (Dr)) ICln used for analysis. IUPred scores are given as mean ± stdev; scores higher than 0.5 indicate disorder.

In intrinsically unstructured proteins or regions within a protein a binding site on a flexible chain could increase the capture radius and facilitate the sampling of a large solution volume for binding targets

(reviewed in [41]). Such a "fishing rod" mechanism was introduced for the unstructured C-terminus of the Shaker potassium channel [42] and discussed for the unstructured distal C-terminus of the Na^+/H^+ exchanger NHE1 [43], and may also apply to ICln which is built up by a N-terminal PH-like domain [19] and an unstructured C-terminus containing protein binding sites.

The C-terminus of ICln is highly conserved in vertebrates (Table 1), suggesting that intrinsic disorder in this part of the protein may be conserved throughout vertebrate evolution. This notion is supported by in silico analysis of protein disorder using the IUPred web server [44], which returned disorder prediction scores almost exclusively higher than 0.5 (indicating disorder) over the entire C-terminus of mammalian, amphibian and teleost ICln (Table 1).

Disordered proteins or regions within a protein may fold into stable secondary or tertiary structures on binding to their targets (reviewed in [45]). As demonstrated here, the region between AA Q159 and H235 in the disordered C-terminus of ICln shows preferred secondary structure elements. Coupled binding and folding into a stable tertiary structure in the presence of an interaction partner like HSPC038 [25] however, could not be demonstrated so far. Also phosphorylation can lead to structure formation in disordered regions of a protein, and may thus regulate binding affinities to partner proteins [46]. Interestingly, it was shown that ICln is phosphorylated *in vivo* by an as yet unidentified kinase [47-48] and that AR2 in the unstructured C-terminus of ICln contains a phosphorylation site [47]. While this finding suggests that ICln interactions and/or function are regulated via posttranslational modification, further studies are needed to determine if phosphorylation plays a role in structure formation and the interaction of ICln with its partner proteins.

In summary, ICln is composed of two domains, a stably folded N-terminal PH-like domain, and a C-terminal domain (AA H134 - H235) which is natively unstructured but shows preferred secondary structure elements (α-helix (AA E170 – E187); β-strands (D161 - Y168 and E217 - T223, respectively)). Knowledge of the structure of ICln may allow studying the function and interactome of the PH-like domain separately from that of the unstructured C-terminus of ICln by a structure guided design of truncation and point mutations.

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