Quaternary Structure Assessment of ICln by Fluorescence Resonance Energy Transfer (FRET) *in vivo*

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Key Words

ICln • Protein-protein interaction • Cell volume • FRET

Abstract

ICln is a ubiquitously expressed multifunctional protein that plays a critical role in regulatory volume decrease after cell swelling. The majority of ICln is localized in the cytosol and a small fraction of ICln associates with the plasma membrane. In artificial lipid bilayers ICln forms ion channels, and a putative channel model predicts the association of at least two ICln molecules to form a functional ion-conducting pore. Oligomers of ICln have been demonstrated in cytosolic fractions of different cells by native PAGE and gel filtration analysis, but these data have not yet been verified *in vivo*, and the basis of ICln homooligomerization is unknown. *In silico* prediction of the quaternary structure of ICln from its primary structure predicts that ICln forms a dimer, and that the C-terminus of ICln may be essential for the intermolecular interaction. To explore the quaternary structure of ICln in living NIH3T3 fibroblasts, we performed fluorescence resonance energy transfer (FRET) experiments using eCFP (donor) and eYFP

(acceptor) fused to the C- and/or N-termini of both full length wild type ICln and of C-terminal truncation mutants thereof (ICln₁₅₉ and ICln₁₃₄). FRET was assessed by the acceptor photobleaching technique. Here we show that ICln forms oligomers *in vivo*, and demonstrate intermolecular FRET between the C-, but not the N-termini of full length ICln. In the truncation mutant ICln₁₅₉ oligomerization occurs and intermolecular FRET between N-termini can be detected, which indicates that the C-terminus of ICln sterically interferes with interactions between N-termini in full length ICln oligomers. In cells expressing the truncation mutant ICln₁₃₄ no FRET between C- and/or N-termini could be measured, suggesting the absence of interaction and a role of amino acids P135-Q159 in the oligomerization of ICln.

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Introduction

ICln is a highly conserved protein expressed in all cells studied so far. Over-expression of ICln in *Xenopus laevis* oocytes leads to the appearance of ion currents with features of the swelling-dependent anion and

osmolyte channel ICln_{swell} [1]. The protein is crucial for regulatory volume decrease (RVD) after cell swelling. Limiting ICln abundance by ICln-specific antibodies, or ICln expression by antisense oligodeoxynucleotides [2, 3] significantly diminishes ICln_{swell}, while increasing ICln activity by over-expression boosts ICln_{swell} activation during hypotonic challenge [4]. Interestingly, over-expression of ICln or ICln-related peptides in *E. coli* improves the tolerance of the bacteria to hypotonicity, an effect that can be reversed by the addition of extracellular nucleotides [5, 6], which are hallmark inhibitors of ICln_{swell} [7]. In addition ICln has been implicated in cell volume-regulated functions like hormone secretion [8].

The proposed role for ICln as a candidate protein making up all or parts of the molecular entity of ICln_{swell} [1] is discussed controversially, since ICln is mainly found as a water-soluble protein in the cytosol, where it interacts with actin [9-11], the non-muscle isoform of the myosin light chain [12], the protein arginine methyltransferase PRMT5 [13], as well as with Sm and LSm proteins [14-17]. Consequently also other functions of ICln have been proposed, for example in the regulation of cell morphology [13], in angiogenesis [18] and most notably in RNA processing [14-17], disclosing its essential role in cell physiology. Accordingly, all attempts to genetically knock out ICln in either mice, nematodes or a cell line failed due to the lethality of the knock-out [19].

While most of ICln resides in the cytosol, a small fraction of it associates with the plasma membrane where interactions with integrin $\alpha_{\text{Ib}}\beta_3$ [20] and the erythrocyte protein p4.1 [21] have been described. During cell swelling a considerable portion of the cytosolic ICln pool travels from the cytosol to the plasma membrane [22-24]. Interestingly, recombinant water soluble ICln is able to insert directly into artificial lipid bilayers to form ion channels [25-29]. On the basis of sequence homology to the pore-forming β -hairpin of α -hemolysin and leukocidin - two water-soluble bacterial toxins that refold and build pores upon insertion into plasma membranes - a putative channel model of ICln was developed that predicts the association of at least two ICln molecules to form a functional ion-conducting pore [1, 25-28]. Multimers of ICln have been demonstrated in the particulate membrane fraction of neonatal rat cardiac myocytes [22] and for recombinant ICln *in vitro* [30]. The structural basis of ICln homooligomerization as well as the presence of ICln homooligomers in living cells, however, has not yet been established.

The aim of the present study was to gain insights into the intra- and intermolecular organization of ICln by

investigating ICln homooligomerization using FRET in living cells.

Materials and Methods

In silico quaternary structure prediction

In silico analysis of ICln homodimerization was performed using the open source Experimental Homodimer Classifier Quaternary Structure Predictor 'Mericity' (<http://www.mericity.com>) [31]. Briefly, the amino acid (AA) sequence of full length ICln and of C-terminal truncations lacking intramolecular domains were submitted to the server and the returned automated categories '0' and '2' were taken as classifiers for non-homodimerization (0) and homodimerization (2), respectively. The estimated 'true' error rate of the predictions is ~30% [31].

Cell culture

NIH3T3 fibroblasts were grown in culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 $\mu\text{g/ml}$ penicillin and 100 U/ml streptomycin in a moist atmosphere at 37°C, 5% CO₂ and 95% air. Subcultures were routinely established on every second to third day of cultivation by trypsin/EDTA treatment (0.05%, pH 7.2).

Transfection procedures

For transfection with the plasmids fibroblasts were grown on glass coverslips (30 mm diameter) to a confluence of approximately 70%. The transfection reagent (polyethylenimine, 22 kDa; ExGenTM, Fermentas, Germany) was used according to the manufacturer's protocol. To scavenge reactive oxygen species generated during the maturation of the enhanced fluorescent proteins, 100 μM of 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TroloxTM, Sigma, Germany), a water-soluble vitamin E analogue, was added to the medium. After 8 hours of incubation the transfection medium was replaced by standard cell culture medium. Transfected cells were used for FRET experiments ~20 hours after replacing the medium. For transfection with two plasmids containing the coding sequences of enhanced cyan fluorescent protein (eCFP) and enhanced yellow fluorescent protein (eYFP), respectively, a molar ratio of eCFP:eYFP of 2:1 was found to yield the best results. The transfection efficiency was approximately 15%.

Salts, chemicals and drugs

All salts, chemicals and drugs were of the purest grade available and purchased from Sigma Aldrich, Germany, unless otherwise stated. Cell culture reagents were obtained from Gibco BRL, Germany.

Preparation of plasmids and fusion proteins

Standard procedures and procedures according to the manufacturers' protocols were used for all DNA preparation-, cloning-, purification- and sequencing steps. Plasmids coding for fusion proteins of canine ICln (NCBI Accession No. CAA46447) and ICln truncations (ICln₁₅₉, truncation of AA

NH2-	MSFLKSFP	PPGSAEGLRQQQPE	TEAV	LNGKGLG	TG	TLYIAE	SR	LSWLD	G	S
51	GL	GFSL	EYPTIS	LHA	VSRDLNAYPREH	LYVMVN	AKFGEESKESVA	EE	ED	S
101	DD	VEPI	IAEFRFVP	SD	KSALEAMFTAMCECQALH		PD	PEDEDSDDYD	GE	EY
151	DVEAHEQGQ		GD	IPTFYTYEEGLSHLTAEGQATLERLEGLMSQSVSSQYNM						
201	AGVRTEDSTRDY		ED	GMEVD	TTPTVAGQFEDADVDH					-COOH

Fig. 1. Amino acid sequence of full length canine ICln and the two different truncations (ICln₁₃₄ and ICln₁₅₉; indicated by vertical red bars) used for the experiments. Beta strands (1-7) and alpha helical structures are indicated by green and blue boxes, respectively. Acidic domains (AD1-3) are given in blue letters.

Fig. 2. Model of FRET between an eCFP-ICln fusion protein as donor and an eYFP-ICln fusion protein as acceptor. If eCFP is excited at 436 nm, energy is transferred from eCFP to eYFP. As a consequence the emission of eCFP at 480 nm decreases and the emission of eYFP at 560 nm increases. FRET can only occur if the two proteins are within a distance of approximately 10 to 90 Å.

G160-H235; ICln₁₃₄ truncation of AA P135-H235; figure 1) with either eCFP or eYFP were prepared by cloning the ORF of ICln in frame to the fluorescent proteins into the vectors pECFP-C1, pEYFP-C1, pECFP-N1 or pEYFP-N1 (Clontech) using standard PCR procedures and XhoI and BamHI restriction sites. Transfection of NIH3T3 fibroblasts with these plasmids results in the expression of fusion proteins of eCFP and/or eYFP, where the fluorescent proteins are attached either to the C-terminus (ICln-CFP, ICln₁₅₉-CFP, ICln₁₃₄-CFP, ICln-YFP, ICln₁₅₉-YFP, ICln₁₃₄-YFP), to the N-terminus (CFP-ICln, CFP-ICln₁₅₉, CFP-ICln₁₃₄, YFP-ICln, YFP-ICln₁₅₉, YFP-ICln₁₃₄), or to both the C- and N-terminus (CFP-ICln-YFP) of full length or truncated ICln, respectively, as shown in table 1. All constructs were verified by sequencing (Microsynth, Switzerland).

construct		description	
1	CFP-ICln-YFP		eCFP attached to the N-terminus and eYFP attached to the C-terminus of ICln
2	CFP-ICln		eCFP attached to the N-terminus of ICln
3	YFP-ICln		eYFP attached to the N-terminus of ICln
4	ICln-CFP		eCFP attached to the C-terminus of ICln
5	ICln-YFP		eYFP attached to the C-terminus of ICln
6	CFP-ICln ₁₅₉		eCFP attached to the N-terminus of ICln ₁₅₉
7	YFP-ICln ₁₅₉		eYFP attached to the N-terminus of ICln ₁₅₉
8	ICln ₁₅₉ -CFP		eCFP attached to the C-terminus of ICln ₁₅₉
9	ICln ₁₅₉ -YFP		eYFP attached to the C-terminus of ICln ₁₅₉
10	CFP-ICln ₁₃₄		eCFP attached to the N-terminus of ICln ₁₃₄
11	YFP-ICln ₁₃₄		eYFP attached to the N-terminus of ICln ₁₃₄
12	ICln ₁₃₄ -CFP		eCFP attached to the C-terminus of ICln ₁₃₄
13	ICln ₁₃₄ -YFP		eYFP attached to the C-terminus of ICln ₁₃₄

Table 1. Fusion proteins expressed in NIH3T3 fibroblasts consisting of eCFP and/or eYFP attached to the N- or C-terminus of full length ICln, ICln₁₅₉ or ICln₁₃₄. Middle row gives schematic illustrations of the single constructs used (Undulated line, ICln; D=donor (eCFP); A=acceptor (eYFP)).

Table 2. Overview of ICln-ICln interactions in living NIH3T3 fibroblasts measured by FRET. eCFP-values are given as percent increase of eCFP-intensity upon 2 minutes of 515 nm acceptor (eYFP) photobleaching (*, $p < 0.05$). n is the number of individual experiments. FRET-efficiency was calculated from the 2-minute eCFP-intensity value as described in *Materials and Methods*. Symbols illustrate the respective constructs (Undulated line, ICln; D=donor (eCFP); A=acceptor (eYFP)).

				Change in eCFP-fluorescence (%)	n	p-value	FRETeff
1	eCFP-photo enhancement	eCFP	D	1.42±0.62	8	0.0556	0.124±0.005
2	Random interaction of plain dyes	eCFP+eYFP	D + A	0.99 ± 0.64	12	0.1524	0.0134 ± 0.004
3	C-terminal interaction	ICln-CFP + ICln-YFP		3.08 ± 0.61*	8	0.0015	0.0387 ± 0.010
4		ICln ₁₅₉ -CFP+ICln ₁₅₉ -YFP		1.77 ± 0.47*	16	0.0164	0.027 ± 0.010
5		ICln ₁₃₄ -CFP+ICln ₁₃₄ -YFP		0.03 ± 0.33	10	0.6470	0.0038 ± 0.002
6	N-terminal interaction	CFP-ICln + YFP-ICln		0.36 ± 0.60	12	0.5590	0.0089 ± 0.003
7		CFP-ICln ₁₅₉ + YFP-ICln ₁₅₉		13.78 ± 2.98*	9	0.0006	0.10 ± 0.016
8		CFP-ICln ₁₃₄ + YFP-ICln ₁₃₄		0.09 ± 0.29	10	0.9360	0.0026 ± 0.001
9	C-N-terminal interaction	CFP-ICln-YFP		4.87 ± 1.02*	12	0.0006	0.0478 ± 0.008
10		ICln-CFP + YFP-ICln		1.96 ± 0.27*	11	0.0001	0.019 ± 0.000
11		ICln ₁₅₉ -CFP + YFP-ICln ₁₅₉		0.91 ± 0.20*	10	0.0083	0.0091 ± 0.002
12		ICln ₁₃₄ -CFP + YFP-ICln ₁₃₄		0.28 ± 0.19	9	0.1491	0.0034 ± 0.002
13		CFP-ICln + ICln-YFP		1.53 ± 0.47*	11	0.0088	0.015 ± 0.004
14		CFP-ICln ₁₅₉ + ICln ₁₅₉ -YFP		0.67 ± 0.33	9	0.0546	0.007 ± 0.003
15		CFP-ICln ₁₃₄ + ICln ₁₃₄ -YFP		0.19 ± 0.23	10	0.9965	0.0039 ± 0.002

Primers

ICln forward primer:

5'-CGA ATT CTC GAG ATG AGC TTC CTC AAA AGT TTC CCG-3' (XhoI)

ICln reverse primer for ICln truncation 159:

5'-CGA ATT CGG ATC CTC ACT GCC CTT GTT CAT GTG CT-3' (BamHI)

ICln reverse primer for ICln truncation 134:

5'-ACA CAG GAT CCT CAA TGC AAG GCC TGG CAT TC-3' (BamHI)

Visualization of eCFP/eYFP-expressing cells and FRET measurements

Transfected cells grown on glass coverslips were mounted in a perfusion chamber and superfused with control solution at room temperature (20-23°C). The control solution was composed of (in mM): NaCl 90, KCl 5, CaCl₂ 2, MgCl₂ 2, glucose 5, mannitol 80, HEPES 10/NaOH (pH 7.4).

For all experiments eCFP served as the FRET donor and eYFP as acceptor as illustrated in figure 2. Using this pair of dyes, FRET can occur if they are in a distance of <10 nm from

each other. Care was taken to choose cells for experiments showing comparable expression levels (based on the fluorescent signal) which are high enough to allow reliable FRET measurements, but as low as possible to minimize potential over-expression artefacts that may influence protein-protein interactions.

Visualization of eCFP and/or eYFP-expressing cells and detection of FRET was performed using a fully automated microscope (iMICTM) equipped with a monochromator (Polychrome VTM), an Imaging System Controller and a cooled charged coupled device (CCD) camera (TILL Imago VGATM) controlled by TILL Vision software (version 4.0). The microscope was operated in the split view mode using a dual excitation filter (transmission ranges 390-450 nm and 490-530 nm, respectively), a multichroic mirror (transmission ranges 445-490 nm and 520-600 nm, respectively) and a dual emission filter (transmission ranges 455-490 nm and 540-590 nm, respectively). eCFP was excited at 436 nm, eYFP was excited and bleached at 515 nm. The emitted light was split by a DichrotomeTM image splitter for simultaneous acquisition of eCFP and eYFP emission images on a single CCD-chip. All components were from Till

Photonics, Germany. All images were adjusted by pixel-to-pixel alignment and corrected for background by subtraction of the mean grey value measured in a cell free area in close proximity of the analysed cells. The background was then clamped to zero by multiplying the background-corrected image by a binary image of the cell, in which all pixels within the cell were set to a value of one and all the pixels outside the cell were set to a value of zero [23]. Images were exported as 16-bit greyscale TIFF files and further processed using the ImageJ software (<http://rsbweb.nih.gov/ij>).

FRET was assessed from the increase of eCFP fluorescence after 2 minutes of acceptor photobleaching. As shown in figures 3 and 6A eYFP is effectively bleached upon illumination at 515 nm. The results are given as relative change of the eCFP-fluorescence intensity after photobleaching (denoted as CFP-gain), as well as the apparent FRET-efficiency ($FRET_{eff}$), which was calculated according to:

$FRET_{eff} = 1 - (CFP^{t0}/CFP^{t2})$, where CFP^{t0} and CFP^{t2} denote the intensities of the eCFP-fluorescence before and after 2 minutes of eYFP photobleaching, respectively.

For $FRET_{eff}$ calculations the integrated grey value of the eCFP-fluorescence intensity within the entire area of the cell was determined. Two by two pixel binning was used to enhance the sensitivity of the CCD and to minimize potential artefacts due to eventual deviations of cell position. In order to test an eventual effect of illumination at 515 nm on eCFP-fluorescence, cells transfected with eCFP alone were subjected to acceptor photobleaching mimicry in the absence of eYFP. Under these conditions eCFP was not bleached within 10 minutes. However, a small transient but statistically not significant photo-enhancement of eCFP was observed after 1 minute (table 2/row 1). Similar results were obtained using CFP-ICln and CFP-ICln₁₅₉, respectively (data not shown). Therefore, although this eCFP-photo-enhancement was not significant, all calculations were made using the values measured 2 minutes after beginning of photobleaching to avoid an eventual overestimation due to a false positive CFP-gain and to prevent erroneous pixel-to-pixel calculations due to eventual deviations of cell position during prolonged observation periods. Given that eYFP is bleached by $90.7 \pm 0.2\%$ ($n=197$) of its initial intensity after 2 minutes, $FRET_{eff}$ values are most likely underestimated. However, as the 2-minute values of eYFP-fluorescence intensity were not statistically significant from the intensity values after 3-10 minutes of photobleaching (figure 3), this error can be neglected and no extrapolation for total eYFP bleaching was performed. The random interaction between free eCFP and eYFP was negligible under the given experimental conditions (table 2/row 2, figure 3). The images shown in figure 5 were acquired from the sensitized eYFP emission after eCFP excitation (NFRET) essentially as described in [23]. Briefly, intensities obtained in the FRET channel (excitation of eCFP at 436 nm, emission of eYFP at 560 nm) were corrected for eCFP cross-talk (contribution of eCFP emission to the eYFP emission window) and eYFP cross-excitation (eYFP emission due to excitation at the eCFP excitation wavelength). The obtained values were normalized for protein expression levels and are referred to as NFRET.

Fig. 3. No significant change in eCFP-fluorescence intensity occurs upon acceptor photobleaching at 515 nm in NIH3T3 fibroblasts expressing plain eCFP and eYFP indicating a negligible random interaction between the dyes under the given experimental conditions. Significant photobleaching of eYFP (*, $p < 0.05$) is evident already after 1 minute and bleached by $90.7 \pm 0.2\%$ ($n=197$) after 2 minutes. No significant further bleaching occurs in the following 8 minutes (ns=not significant).

Statistics

Data are expressed as arithmetic means $\pm$ standard error of the mean (SEM). Statistical analysis was made by paired t-test. Statistically significant differences were assumed at $p < 0.05$.

Results

Quaternary structure prediction

To get first insights into a potential homomultimerization of ICln, we submitted the primary sequences of ICln as well as of the C-terminal ICln truncation mutants ICln₁₅₉ and ICln₁₃₄ to the Experimental Homodimer Classifier Quaternary Structure Predictor 'Mericity'. Dimerization was predicted by 'Mericity' for full length ICln (classification '2', prediction by 2 rules, confidence values 0.958 and 0.738, respectively) as well as for ICln₁₃₄ (classification '2', 1 rule, confidence value 0.786), whereas no homodimerization was predicted for ICln₁₅₉ (classification '0', 1 rule, confidence value 0.9).

FRET analysis

To test a potential homomultimerization of ICln (as determined by 'Mericity') *in vivo*, FRET experiments between fusions of ICln with the fluorescent proteins eCFP (FRET donor) or eYFP (FRET acceptor) were performed in living NIH3T3 cells. Cells were simultaneously transfected with plasmids coding for fusion proteins in which eCFP and eYFP are fused to the C- and/or N-termini of both full length wild type ICln and of

Fig. 4. A Increase of eCFP-fluorescence over time upon acceptor photobleaching at 515 nm in NIH3T3 fibroblasts expressing CFP-ICln-YFP. B Changes in eCFP-fluorescence intensity by acceptor photobleaching of CFP-ICln/YFP-ICln, ICln-CFP/ICln-YFP and CFP-ICln/ICln-YFP expressing NIH3T3 fibroblasts, respectively. C eCFP-fluorescence intensity of CFP-ICln₁₅₉/YFP-ICln₁₅₉, ICln₁₅₉-CFP/ICln₁₅₉-YFP and ICln₁₅₉-CFP/YFP-ICln₁₅₉ expressing fibroblasts. D No significant change in eCFP-fluorescence occurs upon 515 nm eYFP photobleaching in cells expressing ICln₁₃₄-CFP/ICln₁₃₄-YFP, CFP-ICln₁₃₄/YFP-ICln₁₃₄ or CFP-ICln₁₃₄/ICln₁₃₄-YFP, respectively. FRET signals were obtained from whole cells. Symbols illustrate the respective constructs. Undulated line, ICln; D=donor (eCFP); A=acceptor (eYFP).

C-terminal truncation mutants thereof (ICln₁₅₉ and ICln₁₃₄) and FRET demonstrating close proximity of the two fusion proteins was determined by the ‘acceptor photobleaching’ or ‘sensitized emission’ techniques.

In control cells co-expressing plain eCFP and eYFP as separate proteins, no significant FRET could be detected, excluding that FRET signals can arise from a random interaction of the donor and acceptor (table 2/row 2).

Interactions of full length ICln

To test if FRET between eCFP and eYFP can occur when the fluorescent proteins are fused to the opposite ends of ICln, i.e. the N- and C-terminus, CFP-ICln-YFP (table 1) was expressed in the cells. In this construct ICln serves as a ‘spacer’ between eCFP and eYFP. As shown in table 2/row 9, as well as figures 4A and 5A, significant FRET can be measured, demonstrating that the N- and C-termini of ICln are in a proximity of <10 nm either within a single ICln molecule (i.e. intramolecular interaction) or between different ICln molecules (i.e. intermolecular interaction) to allow for FRET.

To test the latter possibility, i.e. if ICln molecules interact with each other, eCFP and eYFP were fused either to the C-terminus (ICln-CFP, ICln-YFP; table 1) or N-terminus (CFP-ICln, YFP-ICln, table 1) of ICln. Significant FRET was measured in cells co-expressing

CFP-ICln + ICln-YFP (table 2/row 13 and figure 4B) and ICln-CFP + YFP-ICln (table 2/row 10) indicating that ICln forms multimers in which N- and C-termini of separate ICln molecules are located within <10 nm. FRET was also apparent in cells expressing ICln-CFP + ICln-YFP (table 2/row 3, figures 4B and 5B), but not in cells co-expressing CFP-ICln + YFP-ICln (table 2/row 6, figures 4B and 6A). This indicates that in full length ICln interaction can occur between the C-termini, the C- and N-termini, but not between the N-termini.

Interactions of ICln₁₅₉

To narrow down the region needed for the ICln homooligomerization and to test whether the C-termini might affect possible N-terminal interactions, we truncated the 76 C-terminal amino acids (AA G160-H235) of ICln (ICln₁₅₉) and fused either eCFP or eYFP to the N- or C-terminus, respectively, of this ICln truncation mutant. When CFP-ICln₁₅₉ + YFP-ICln₁₅₉ were co-expressed, strong FRET was observed (table 2/row 7, figures 4C and 6B), indicating N-N-terminal interactions in the absence of AAs G160-H235. The significantly higher FRET between CFP-ICln₁₅₉ + YFP-ICln₁₅₉ as compared to CFP-ICln + YFP-ICln suggests that in multimers of full length ICln, the N-termini of individual ICln molecules are located too far apart to yield FRET.

Significant FRET was also observed between

Fig. 5. A Sensitized eYFP emission after eCFP excitation measured in NIH3T3 fibroblasts expressing CFP-ICln-YFP. The FRET signal corrected for eCFP cross-talk and eYFP cross-excitation is normalized for eCFP and eYFP expression levels (NFRET) as described in [23]. B Transmission image and YFP fluorescence (upper panels) and NFRET (lower panel) of fibroblasts cotransfected with ICln-CFP and ICln-YFP. To better visualize intensity differences, images were pseudocolored. Calibration bars show the relative degree of NFRET. Symbols illustrate the different constructs used. Undulated line, ICln; D=donor (eCFP); A=acceptor (eYFP).

ICln₁₅₉-CFP + ICln₁₅₉-YFP (table 2/row 4, figure 4C), between CFP-ICln₁₅₉ + ICln₁₅₉-YFP (table 2/row 14, figure 4C) as well as between ICln₁₅₉-CFP + YFP-ICln₁₅₉ (table 2/row 11, figure 4C), indicating C-C terminal- and C-N terminal interactions of the truncated proteins, respectively.

Interactions of ICln₁₃₄

To further delineate the region essential for ICln homooligomerization, we next truncated further 25 AAs from the C-terminus of ICln₁₅₉, leading to the ICln

Fig. 6. A FRET-images acquired in living NIH3T3 fibroblasts expressing CFP-ICln and YFP-ICln. B Images acquired in NIH3T3 fibroblasts expressing CFP-ICln₁₅₉/YFP-ICln₁₅₉. Panels a and b show the eCFP-fluorescence (ex. 436 nm, em. 480 nm) before (CFP min 0) and after 2 minutes (CFP min 2) of acceptor photobleaching at 515 nm, respectively. Panels c and d show the eYFP-fluorescence (ex. 515 nm, em. 560 nm) before (YFP min 0) and after (YFP min 2) acceptor photobleaching, respectively. Panels e are the respective FRET-efficiency (FRET_{eff}) images calculated as described in *Materials and Methods*. Note the strong increase of eCFP-fluorescence in B and the difference between the FRET_{eff} images. For better visualization pseudocolors were applied. Calibration bars represent the relative degree of fluorescence intensity or FRET_{eff}. Symbols illustrate the different constructs used. Undulated line, ICln; D=donor (eCFP); A=acceptor (eYFP).

truncation mutant ICln₁₃₄ and fused either eCFP or eYFP to either the N- or the C-terminus of this ICln truncation mutant. ICln₁₃₄ lacks the entire C-terminus (AAs P135-H235) of ICln and is thus built up by AAs M1-H134 which are structured in a PH domain like fold [32].

In none of the cells expressing ICln₁₃₄, FRET could be detected, regardless of the combinations tested (figure 4D), i.e. ICln₁₃₄-CFP + ICln₁₃₄-YFP (table 2/row 5), CFP-ICln₁₃₄ + YFP-ICln₁₃₄ (table 2/row 8), ICln₁₃₄-CFP + YFP-ICln₁₃₄ (table 2/row 12) or CFP-ICln₁₃₄ + ICln₁₃₄-YFP (table 2/row 15).

Discussion

ICln is an acidic protein that is structured in two domains. The N-terminus (AAs M1-H134), whose structure was determined by NMR measurements, shows a PH-domain-like fold where the N- and C-termini are roughly located on opposite sides of the truncated molecule [32] and which contains the putative pore forming part of ICln [1, 25]. The C-terminal structure (AAs P135-H235) has not been determined yet. This C-terminus of ICln, which contains the acidic domains 2 and 3, is most likely an interaction- and/or regulatory part of the protein, as it contains binding sites for partner proteins like LSm4 [32], PRMT5 [12, 13, 16], the erythrocyte protein 4.1 [21] or kinases [33, 34]. ICln is assumed to interact with other ICln molecules to form homooligomers [22, 30]. The structural basis of ICln homooligomerization as well as the presence of ICln homooligomers in living cells, however, has not yet been established.

As an *in silico* approximation of a potential homooligomerization of ICln we analysed the propensity of ICln to form dimers by submitting the AA sequence of full length ICln to the experimental homodimer classifier 'Mericity', which predicts the formation of full length ICln homodimers. To narrow down the regions important for the putative homodimerization, primary sequences of truncation mutants of ICln (ICln₁₅₉ and ICln₁₃₄) that had previously been characterized on a structural and functional level [32], were also submitted. Dimerization is predicted for ICln₁₃₄, but not for ICln₁₅₉, suggesting that ICln can form dimers presumably by the N-terminal interaction with a crucial role of the C-terminus in governing this process.

As a first *in vivo* experimental approach to test these predictions we probed for FRET between eCFP- and eYFP-labeled N- and/or C-termini of ICln by expressing different combinations of these fusion proteins (shown in tables 1 and 2). In a system expressing exclusively dye-labeled ICln, FRET is determined by the donor-acceptor distance and by the orientation of the fluorescent dyes to each other [35]. It is hence dependent on the primary AA sequence length, the globularity and the intramolecular mobility of the molecule as well as on the number and the stochastic arrangement of the interacting ICln fusion proteins. Since the transfected NIH3T3 fibroblasts also express endogenous, i.e. unlabeled ICln, the efficiency of FRET in this *in vivo* system is in addition dictated by the degree and stochastic arrangement of a potential oligomerization of endogenous ICln with the heterologously expressed labeled ICln species. Such an ICln 'hetero-

oligomerization' - whose degree cannot be determined in the experimental system employed - is expected to affect the true efficiency of ICln-ICln FRET as well. In addition it cannot be excluded that the presence of the FRET probes alters the association properties in the ICln fusion proteins tested. Therefore, the apparent values of FRET-gain and FRET-efficiency are likely to underestimate the true degree of ICln-ICln oligomerization. The low FRET signals observed in our experiments may therefore be explained by (i) a long distance between the N- and C-termini of ICln and/or (ii) a specific orientation of the donor-acceptor pair and/or (iii) by ICln 'heterooligomerization' and/or (iv) formation of 'blind' homomultimers consisting of ICln molecules labeled with the same fluorescent protein and/or (v) altered association properties of ICln-fusion proteins.

To test for intramolecular C-N-terminal interactions, we transfected cells with the CFP-ICln-YFP construct, in which eCFP and eYFP are fused to the N- and C-terminus, respectively, so that ICln serves as a 'spacer' between donor and acceptor. The occurrence of FRET in this approach indicates intramolecular C-N-terminal interactions. However, the FRET signals measured using the CFP-ICln-YFP fusion protein could also arise from C-N-terminal intermolecular ICln-ICln interactions, i.e. from homooligomerization. Therefore we next studied the formation of ICln homomers by co-expressing full length ICln fused to either eCFP or eYFP to the C- or N-terminus of ICln, respectively. In all combinations tested, only the one in which the fluorescent proteins are fused to the N-terminus of full length ICln, i.e. the FRET pair CFP-ICln + YFP-ICln, did not yield significant FRET. FRET occurred between the FRET pairs ICln-CFP + ICln-YFP, CFP-ICln + ICln-YFP and ICln-CFP + YFP-ICln. These findings clearly indicate that ICln forms homooligomers *in vivo*, that these homooligomers allow C-C-terminal and C-N-terminal FRET and that the N-termini of individual ICln molecules within the full length ICln homomers are too far apart to yield FRET or interfere with the formation of homomultimers.

Interestingly, all FRET pairs of the C-terminal truncation mutant ICln₁₅₉ (CFP-ICln₁₅₉ + YFP-ICln₁₅₉, CFP-ICln₁₅₉ + ICln₁₅₉-YFP and ICln₁₅₉-CFP + ICln₁₅₉-YFP) produced significant FRET. Notably, in contrast to full length ICln (CFP-ICln + ICln-YFP, which did not yield FRET), the highest FRET signals observed in this series of experiments were produced by CFP-ICln₁₅₉ + YFP-ICln₁₅₉, indicating that N-terminal interactions of ICln can occur in the absence of the C-terminal AAs G160 to H235 and that the lack of N-N-terminal FRET

in full length ICln is unlikely to be due to interference of the fluorescent proteins with homooligomer formation. No dimerization was predicted for ICln₁₅₉ by *in silico* analysis (see above), and in solution NMR measurements using ICln₁₅₉ *in vitro* homooligomerization was not observed [32]. Obviously this homooligomerization does not occur spontaneously but requires the intact environment of the living cell.

Despite *in silico* prediction for N-terminal homodimerization and the measured N-N-terminal interaction, no FRET was observed in cells expressing any of the fluorescent fusion proteins of the truncation mutant ICln₁₃₄, including the FRET pair CFP-ICln₁₃₄ + YFP-ICln₁₃₄ which would be expected to produce measurable FRET, if the domain enabling the N-N-terminal interactions observed was located at the N-terminus of ICln itself. This finding therefore suggests that AAs P135-Q159, which contain the acidic domain 2, are involved in the formation of ICln homooligomers. We can, however, not exclude the possibility that fusion of fluorescent proteins to this shorter ICln truncation mutant per se impedes the formation of ICln multimers as mentioned above. However, in line with this finding and in contrast to wild type ICln, over-expression of the ICln truncation mutant ICln₁₃₄ does not augment ICls_{well} in hypotonic conditions in cells and recombinant purified ICln₁₃₄ does not induce ion currents in lipid bilayer experiments (M. P., personal communication). Since it is established that in isotonic conditions ICln is part of different multi-subunit protein complexes containing splicing factors amongst others [14-17], homomultimerization may also occur in these complexes and be unrelated to channel formation of ICln.

From the present experiments it cannot be determined whether the ICln homooligomerization is restricted to defined cellular compartments, e.g. to the cytosol or the cell membrane. This has to be determined in future studies.

In conclusion our data suggest that (i) ICln can form homomultimers *in vivo*, (ii) the C- and N-terminal ends of ICln undergo inter- and/or intramolecular interactions, (iii) the N-termini of ICln cannot interact with each other in the presence of the complete C-terminus, and (iv) the deletion of parts of the C-terminal part including the acidic domain 3 gives rise to N-N-terminal interaction. Since deletion of AAs containing the acidic domains 2 and 3 annihilates FRET, these domains seem to be necessary for homooligomerization in living cells.

Abbreviations

AA (amino acid); eCFP (enhanced cyan fluorescent protein); eYFP (enhanced yellow fluorescent protein); EDTA (ethylenediaminetetraacetic acid); FRET (fluorescence resonance energy transfer); FRET_{eff} (FRET efficiency); HEPES (N-2-hydroxyethyl-piperazine-N'-2 ethanesulfonic acid); ICls_{swell} (swelling-dependent chloride channel/current); NFRET (net-FRET normalized for eCFP- and eYFP expression); net-FRET (eYFP-emission after eCFP excitation corrected for eYFP donor cross-excitation and eCFP-eYFP-spectral cross-talk).

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