CARP interacts with titin at a unique helical N2A sequence and at the domain Ig81 to form a structured complex

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The cardiac ankyrin repeat protein (CARP) is up-regulated in the myocardium during cardiovascular disease and in response to mechanical or toxic stress. Stress-induced CARP interacts with the N2A spring region of the titin filament to modulate muscle compliance. We characterize the interaction between CARP and titin-N2A and show that the binding site in titin spans the dual domain UN2A-Ig81. We find that the unique sequence UN2A is not structurally disordered, but that it has a stable, elongated α -helical fold that possibly acts as a constant force spring. Our findings portray CARP/titin-N2A as a structured node and help to rationalize the molecular basis of CARP mechanosensing in the sarcomeric I-band.

Keywords: circular dichroism; recombinant proteins; SEC-MALLS; small-angle X-ray scattering; X-ray crystallography

The muscle protein titin (> 38 000 residues) is a central player in cardiovascular health and disease [1,2]. Titin contributes to maintain the mechanical properties of muscle and to the functional integration of sarcomere signaling. Two important signaling hubs have been identified in the elastic I-band region of titin: the N2A and the N2B elements [1,3,4]. N2A is functionally essential and is present in both cardiac and skeletal isoforms of titin, whereas N2B is found exclusively in the heart. N2A interacts with the calcium-dependent proteases, calpains [5,6], and with MARPs, a family of muscle-specific ankyrin repeat proteins [7,8]. The importance of the N2A element is demonstrated by a

deletion that truncates domain Ig83 and which causes severe muscular degeneration in *mdm* (muscular dystrophy and myositis)-mutant mice [9]. Here, calpain-3 binding is abolished [5] and MARP binding is abnormally up-regulated, pointing to the central role of N2A in modulating both signaling pathways [10].

The MARP gene family comprises three members: the cardiac-specific CARP/Ankrd1, Ankrd2/Arpp, and the diabetes-associated DARP [7]. While MARPs are not generally essential [11], the CARP isoform is particularly relevant as its expression in the heart is consistently up-regulated during heart failure. Initially, CARP was identified as a nuclear regulator of

Abbreviation

AR, ankyrin repeats; BiYFP, bimolecular fluorescence complementation of yellow fluorescent protein; CARP, cardiac ankyrin repeat protein; CD, circular dichroism; DRI, differential refractive index; DSF, differential scanning fluorimetry; MM, molecular mass; NRMSD, normalized root-mean-square deviation; R_g , radius of gyration; SAXS, small-angle X-ray scattering; SEC-MALLS, size exclusion chromatography with multi-angle laser light scattering; TEV, tobacco etch virus.

the Nkx2.5 pathway [12,13], but subsequent studies also demonstrated extranuclear roles, including a stress-induced targeting of the sarcomere, e.g., in response to mechanical overload [7,14]. CARP is also deregulated in heart failure of diverse origins, including dilated cardiomyopathy, hypertrophic cardiomyopathy, and arrhythmogenic right ventricular cardiomyopathy [15–18]. In all cases, its presence correlates with disease progression allegedly as a result of playing a role in disease resistance [19]. Thus, CARP is currently viewed as a stress response factor in the heart, where it appears to increase the resistance of cardiomyocytes to apoptosis, protecting the myocardium against damage.

Upon its stress induction, CARP localizes largely to the sarcomeric I-band, where it interacts with titin N2A [7,11]. As the N2A segment is an elastic spring of titin, its interaction with CARP is thought to modulate muscle compliance. Such modulation might be induced partly by a direct alteration of the stretch response of the spring and partly by the protection of N2A against phosphorylation. N2A is phosphorylated by PKA (or PKG), which increases titin's compliance and reduces passive force in the sarcomere [8,20]. Accordingly, myofibrils from knockout mice of CARP, MARP, and DARP show high levels of phosphorylation and become more compliant as the length of the myofibril increases [21], supporting the view that CARP can control the stiffness of titin by blocking N2A phosphorylation. In addition, it has been hypothesized that CARP acts as a cross-linker of titin filaments, providing stretch resilience to the sarcomere [8]. As stretch overload causes apoptosis of cardiomyocytes, leading to heart failure [22], it is speculated that the CARP : N2A interaction might have an antiapoptotic role in muscle.

At the molecular level, CARP consists of N- and C-terminal domains. The N-terminal fraction (~115 residues) is predicted as structurally disordered [8]. However, it contains a short coiled-coil motif that induces the antiparallel dimerization of CARP, according to bimolecular fluorescence complementation of yellow fluorescent protein (BiYFP) and pull-down assays [8,23]. CARP's C-terminal region (~200 residues) is predicted to fold into various ankyrin repeats (AR; 4–6 repeats variably predicted; [8,24,25]). This AR domain has been shown to bind titin N2A [8]. The latter consists of a unique sequence and four Ig domains (Fig. 1). Data from yeast two-hybrid screens indicated that the elastic unique sequence between Ig80 and Ig81, UN2A, is the site of interaction with CARP [7].

Despite the importance of CARP-N2A signaling in heart physiology, little is known about the molecular basis of this interaction. Here, we characterize bio-physically the CARP : N2A complex and its

Fig. 1. Domain composition of the N2A region of titin. The composition of the various constructs used in this study is shown.

components and provide an experimental foundation that can support future studies on the function of this association.

Methods

Expression clones

CARP^{106–319} (UniProtKB Q15327) and the domain variants of titin N2A (UniProtKB Q8WZ42; Fig. 1) were cloned into the pET-trx1a vector. This vector fuses a His₆-tag, a thioredoxin domain, and a tobacco etch virus (TEV) protease cleavage site to the N terminus of the inserted gene. All plasmids were confirmed by sequencing.

Recombinant protein production

Proteins were expressed in *E. coli* Rosetta (DE3; Merck Millipore, Billerica, MA, USA) cultivated in Luria–Bertani media supplemented with 25 µg mL^{−1} kanamycin and 34 µg mL^{−1} chloramphenicol at 37 °C until OD₆₀₀ = 0.6. Protein expression was induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside and cultures grown further overnight at 18 °C. Cells were harvested by centrifugation and lysed with a pressure homogenizer in 25 mM HEPES pH7.5, 300 mM NaCl in the presence of an EDTA-free protease inhibitor cocktail (Roche Applied Science, Penzberg, Bavaria, Germany) and 1 mg DNase (Sigma-Aldrich, St. Louis, MO, USA). The lysate was clarified by centrifugation. The purification of all proteins from supernatants followed Ni²⁺-NTA affinity chromatography, tag removal by TEV protease digestion, reverse affinity chromatography, and size exclusion chromatography – the latter in 25 mM HEPES pH7.5, 100 mM NaCl. Purified samples were stored at 4 °C until further use.

CARP : titin complexes were produced by mixing purified samples at 1 : 1 molar ratio in 25 mM HEPES pH7.5, 100 mM NaCl.

Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded on an 1100 CD spectrometer (JASCO, Easton, MD, USA). Samples

were studied in 20 mM sodium phosphate pH7.5 at a concentration of 0.18 mg mL⁻¹ for CARP^{106–319} and 0.29 mg mL⁻¹ for UN2A. The spectral range was $\lambda = 185\text{--}260$ nm and a 1 mm cell (cell type 110-QS; HELIMA, Müllheim, Germany) was used. Spectra were acquired three times and averaged. The CDSSTR program [26] in Dichroweb (<http://dichroweb.cryst.bbk.ac.uk>) was used to estimate secondary structure content. The fit between experimental and reconstructed spectra was evaluated by their normalized root-mean-square deviation (NRMSD) (NRMSD > 0.05 signifies a nonacceptable fit).

For thermal denaturation, CARP^{106–319} was assayed in the temperature range 25–80 °C and UN2A between 20 °C and 95 °C. In both cases, the temperature increase rate was 1 °C min⁻¹ with an incubation time of 90 s at each integral degree. Each spectrum was collected twice and averaged. Thermal denaturation curves were calculated at $\lambda = 208$ nm for CARP^{106–319} (as this was the region of larger spectral change) and $\lambda = 220$ nm for UN2A, and the melting temperature T_m obtained from the first derivative.

Size exclusion chromatography with multiangle laser light scattering

The molecular mass (MM) of protein samples was determined by size exclusion chromatography with multiangle laser light scattering (SEC-MALLS) on an ÄKTA pure (GE healthcare, Little Chalfont, UK) connected to a MALLS detector and a differential refractive index (DRI) detector (DAWN HELEOS-II and Optilab TrEX; Wyatt Technology, Santa Barbara, CA, USA). The proteins were in a 25 mM HEPES pH7.5, 100 mM NaCl solution that had been three times filtrated (0.22 µm filter) and degassed. All measurements were on a HiLoad Superdex75 10/300 column (GE healthcare) running at a 0.75 mL min⁻¹ flow rate. The total amount of protein injected was ~1 mg (at an approximate concentration of 2 mg mL⁻¹). The system was calibrated using bovine serum albumin prior to the measurements.

NMR spectroscopy

2D TOCSY (mixing time 40 ms) and NOESY (mixing time 100 ms) spectra from unlabeled UN2A sample at 15 mg mL⁻¹ were collected at 298K in 25 mM HEPES pH7.5, 100 mM NaCl, 3 mM β-ME, 5% [v/v] ²H₂O on an Avance III 600 MHz spectrometer equipped with CryoProbe (Bruker, Karlsruhe, Germany). Spectra were processed with TopSpin (Bruker) and referenced to the external standard trimethylsilylpropanoic acid.

Small-angle X-ray scattering

Small-angle X-ray scattering (SAXS) data were collected at the B21 beamline of the Diamond Light Source synchrotron (Didcot, UK) using the integrated SEC-SAXS

setup including the HPLC device Agilent 1260C (Santa Clara, CA, USA). Protein samples (45 µL volume) at a concentration of 13.3 mg mL⁻¹ UN2A, 13.4 mg mL⁻¹ UN2A-Ig81, 14.9 mg mL⁻¹ CARP : UN2A-Ig81 were injected onto a pre-equilibrated Shodex KW403 column (25 mM HEPES pH7.5, 100 mM NaCl) and SEC performed at a flow rate of 0.16 mL min⁻¹. Frames were collected for the entire eluate using an exposure time of 3s per frame and a sample cell thermostated to 20 °C. X-ray scattering was recorded on a Pilatus 2M detector (Dectris, Baden-Daettwil, Switzerland) at a sample to detector distance of 3.9 m and a $\lambda = 1$ Å. Data processing used SCATTER [27]. Scattering curves were analyzed with SCATTER and PRIMUS [27,28] to determine the radius of gyration (R_g), the maximum dimension (D_{max}), the pair distribution function $P(r)$, and the experimental MM. Low-resolution *ab initio* models were calculated without the imposition of symmetry restraints using DAMMIF [29]. Twenty independent models were generated and averaged with DAMMAVER [30] and subjected to final refinement in DAMMIN [31].

Crystal structure determination of domain Ig81

Domain Ig81 was crystallized using a nanovolume dispenser robot (Innovadyne, Santa Rosa, CA, USA) on 96-well Intelliplates using a sitting drop setting at 19 °C. Crystals grew from solutions containing 0.2 M MgCl₂, 0.1 M Tris pH 8.5, 30% [w/v] PEG 4000. Ig81 was at a concentration of 38.5 mg mL⁻¹. The precipitant : protein drop ratio was 200 : 100 nL. Crystals were vitrified in LN₂ in cryo-protection medium (20% [v/v] ethylene glycol, 20% [v/v] isopropanol, 0.2 M MgCl₂, 0.1M Tris pH8.5) and used in data collection. X-ray diffraction data were processed using XDS/XSCALE [32] (Table 1). Phasing was by molecular replacement in PHASER [33] using Ig10 from titin (PDB 4QEG) as search model. The model of Ig81 was created with automatic model building in ARP/WARP [34] and manual rebuilding in COOT [35]. Model refinement used PHENIX.REFINE [36].

Results

The ankyrin repeat domain of CARP forms dimers with high helical content

CARP binds titin N2A via its C-terminal AR domain [8]. To study this interaction, we created an N-terminally truncated CARP construct corresponding to the titin-binding domain, CARP^{106–319}. The overexpression of CARP^{106–319} in *E. coli* yielded 7 mg of purified sample per 1 L culture (Data S1). Analysis of CARP^{106–319} by SEC-MALLS resulted in a measured MM of 48.6 kDa (Table 2; Data S2), indicating that CARP^{106–319} forms dimers (MM calculated from

Table 1. X-ray data processing statistics and model refinement parameters. EDO, ethylene glycol; IPA, isopropanol.

	Ig81
PDB code	5JOE
Space group	$P6_522$
Cell dimensions	
a, b, c (Å)	115.31, 115.31, 52.23
α, β, γ (°)	90, 90, 120
Solvent content (%)	43
Matthews coefficient (Å ³ /Da)	2.4
Copies in asymmetric unit	1
X-ray data	
X-ray source	DIAMOND I04-1
Detector	PILATUS 6M-F
Wavelength (Å)	0.9282
Resolution (Å)	28.83–2.0 (2.05–2.0)
Unique reflections	14 310 (993)
Multiplicity	11.47 (11.67)
Completeness, %	99.8 (98.6)
$\langle I/\sigma(I) \rangle$	23.28 (2.87)
R_{sym} (I)	10.4 (87.9)
CC (1/2)	99.9 (86.5)
Refinement	
No. of reflections	14 290/430
Working/test set	
Protein atoms/waters	765/179
Ligands	EDO × 2, IPA × 1
$R_{\text{factor}}/R_{\text{free}}$, %	0.1775/0.2184
RMSD bond length, Å	0.003
RMSD bond angle, °	0.71

sequence is 24.1 kDa). This result was unexpected as CARP^{106–319} lacks the N-terminal coiled-coil motif thought to be indispensable for CARP dimerization [8]. Our findings reveal now that the AR domain can contribute to the N terminus-led dimerization of CARP.

Next, we estimated the secondary structure content of CARP^{106–319} using CD spectra. These calculations indicated that CARP^{106–319} contains approximately 41% helical and 13% β -turn elements (NRMSD = 0.03; Fig. 2C). The result is in excellent agreement with secondary structure predictions performed using PSIPRED (<http://bioinf.cs.ucl.ac.uk/psipred/>; [37]), Jpred4 (<http://www.compbio.dundee.ac.uk/jpred/>; [38]), and PCI-SS (<http://bioinf.sce.carleton.ca/PCISS/start.php>; [39]). Predictions consistently suggested the

Table 2. Molecular mass analysis of CARP and N2A samples.

Sample	Sequence (MM; kDa)	SEC-MALLS (MM; kDa)	Oligomeric state
CARP ^{106–319}	24.1	48.6	Dimer
N2A	33.9	36.0	Monomer
UN2A	13.3	13.1	Monomer
CARP ^{106–319} : N2A	58.0	59.4	1 : 1

presence of 12 short helices indicative of the existence of six AR motifs, amounting to ~ 40% helical content. AR motifs contain ~ 33 residues that fold into two short α -helices linked by a tight turn, forming a helix-loop-helix α -hairpin structure where each adjacent α -helix is arranged in an antiparallel fashion [40]. AR units pack consecutively into an array, being linked by a loop of variable length that projects outward from the helices at a 90° angle. Upon stacking of the repeats, the fold adopts a slightly concave shape. Although there is no strict sequence conservation in AR motifs, their composition approximates the consensus ‘G-TPLHhA---G-h[-]hh-h--GA-h-A[+]’, where h indicates a hydrophobic residue and [-] and [+] negatively and positively charged residues, respectively [41]. Prominent features are the conserved TPLH sequence in the first α -helix that is involved in a tight network of hydrogen bonds of structural importance and the LL-GA motif at the end of each individual AR. The TPLH feature is clearly recognizable in each of the six predicted AR in CARP^{106–319} (Fig. 2A). Thus, taking CD data and identifiable sequence motifs together, we concluded that the C-terminal domain of CARP contains six AR motifs. In agreement, a representative 3D-model of CARP^{106–319} calculated independently with I-TASSER [42] contained six AR motifs (Fig. 2B).

The stability of the CARP^{106–319} fold was then estimated by thermal denaturation. For this, changes in the CD spectrum upon temperature increase were used to calculate the melting temperature (T_m) of the domain. This yielded a T_m value of 43 °C (Fig. 2D). The result was confirmed using differential scanning fluorimetry (DSF), which exactly reproduced the value (Data S3).

The unique titin N2A domain has a helical and thermally stable fold

In titin, the unique sequence UN2A has been reported as the CARP-binding site [8]. UN2A is believed to be intrinsically disordered (e.g., [8]). In support of this view, the disorder predictor PONDR-fit [43] suggested notable portions of disorder in the N- and C-terminal fractions of UN2A, with only a small central region proposed as structured. In contrast, disorder predictors DISEMBL [44], GLOBPLOT2 [45], and PREDICT PROTEIN [46] did not consistently identify any areas of significant disorder. The latter agrees with secondary structure predictions from PSIPRED [37], Jpred4 [38], and PCI-SS [39] that estimate that UN2A has an α -helix rich secondary structure (approximately 60–70% helical content; Fig. 3A).

Fig. 3. Analysis of the UN2A fold. (A) Secondary structure prediction of UN2A from sequence data using the PCI-SS server [39]. Boxed (blue) is a sequence segment in the central portion of UN2A with shared similarity to a helical motif from MyBP-C3; (B) Sequence alignment of the segment common to MyBP-C3 and UN2A where conserved groups are indicated. The NMR structure of the MyBP-C3 fragment (PDB 2LHU) is shown and the hydrophobic residues conserved in UN2A explicitly displayed. The conservation of these groups suggest a similar helical packing of this segment of UN2A; (C) CD spectra of UN2A recorded in 20 mM sodium phosphate buffer pH7.5 at 25 °C (Ellipticity); (D) $^1\text{H}/^1\text{H}$ region of 2D NOESY spectrum showing a large number of contacts between backbone ^1H protons characteristic of helical secondary structure.

time. This deduction is strongly supported by the elution profile of UN2A in SEC, where UN2A elutes at much lower exclusion volume than expected for a protein of this MM (Data S5), indicative of a highly anisometric molecular shape. Taken together, NMR and SEC data suggested at this stage that UN2A is likely to adopt an elongated helical fold.

Finally, we estimated the stability of the UN2A fold by CD-monitored thermal denaturation. Unexpectedly, the temperature increase only yielded a small, noncooperative change in CD signal, which did not allow calculating a T_m value for UN2A (Fig. 3C). We then incubated UN2A samples at 95 °C for 90 s and at 99 °C for 10 min. In both cases, UN2A retained high

levels of secondary structure (Data S6). This suggested that UN2A has a stable fold and that its helices are not merely transient formations.

CARP forms a robust complex with the dual domain UN2A-Ig81 segment of titin

To validate UN2A as the CARP-binding locus of titin N2A, we tested the binding of the recombinant CARP^{106–319} and UN2A samples using SEC. However, only a modest cosegregation of the samples could be observed (Fig. 4B), indicating that the complex forms only weakly. We then extended the UN2A construct to include its flanking domains: constructs Ig80-UN2A

Fig. 4. Analysis of the complexation of CARP and N2A species. Complexes of CARP¹⁰⁶⁻³¹⁹ and (A) UN2A, (B) Ig81, and (C) UN2A-Ig81 mixed at 1 : 1 molar ratio studied by SEC using a same Superdex75 16/60 column (GE Healthcare) in 25 mM HEPES pH7.5, 100 mM NaCl. Only UN2A-Ig81 yields a strong cosegregation with CARP¹⁰⁶⁻³¹⁹.

and UN2A-Ig81 were generated. Ig80-UN2A could not be produced solubly in *E. coli*, but UN2A-Ig81 was obtained in a yield of 10 mg per 1 L culture (Data S1). SEC showed that the interaction between UN2A-Ig81 and CARP^{106–319} is robust, with a clear cosegregation of species permitting the isolation of the complex (Fig. 4A). Hence, we deduced that Ig81 must be part of the CARP-binding site in titin. However, when we tested the complexation of CARP^{106–319} with the isolated Ig81, no cosegregation was observed suggesting that Ig81 on its own does not interact detectably (Fig. 4C). We thus concluded that Ig81 and UN2A cooperate in forming a shared binding site for CARP.

Binding to titin disrupts the dimeric state of the ankyrin repeat domain of CARP

As CARP^{106–319} forms dimers but N2A components are monomeric (Table 2), we studied the stoichiometry of the complex using SEC-MALLS. To better resolve complexed and single species, we used an extended segment of titin N2A comprising Ig80-UN2A-Ig81, here termed N2A (Fig. 1). N2A was produced recombinantly with a yield of 8 mg of pure protein per 1 L culture (Data S1). The analysis of the CARP^{106–319} : N2A complex by SEC-MALLS yielded a MM of 59.4 kDa (Table 2; Data S2). As the theoretical MM of CARP^{106–319} is 24.1 kDa and that of N2A is 33.9 kDa, the measured MM is in excellent agreement with a 1 : 1 CARP^{106–319} : N2A complex. This implies that the CARP^{106–319} dimer interface must be disrupted by its binding to titin N2A, so that N2A binding is in competition with AR-mediated CARP dimerization.

Biophysical characterization of the CARP : titin complex and its components

We studied the overall molecular features of UN2A, UN2A-Ig81, and the CARP : UN2A-Ig81 complex using SAXS (Fig. 5; Table 3). MM estimates indicated that all samples are predominantly monomeric in solution, agreeing with SEC-MALLS data. The experimental R_g and D_{max} values pointed to elongated molecular shapes for all constructs, but specially UN2A and UN2A-Ig81. Specifically, the R_g and D_{max} values of UN2A (21.7 Å and 9.0 nm, respectively) were roughly twice those of a globular protein of same mass (13.5 Å and 3.5 nm, respectively, for a hydrated spherical protein particle of density 1.35 g cm^{−3}; $R_g^2 = 3/5 \cdot r^2$). The elongated shape of these samples was also confirmed by the overall appearance of the pair distance distribution function $P(r)$ that defines the frequency of distances r joining two volume elements in the molecule.

The $P(r)$ functions of UN2A and UN2A-Ig81 displayed a skewed appearance characteristic of elongated structures, and exhibited a tail indicative of an extended protrusion (Fig. 5). The marked tail of the $P(r)$ function further points to a modest internal dynamics in these samples, as marked motions would result in a disappearance or smearing of this feature.

The interpretation of SAXS data in terms of low-resolution molecular shapes resulted in elongated structures for UN2A and UN2A-Ig81 (Fig. 5), consistent with deductions above. The *ab initio* model of the CARP : UN2A-Ig81 complex suggested the binding of CARP in transversal orientation at the junction of the UN2A and Ig81 domains (Fig. 5), in agreement with expectations.

The crystal structure of Ig81 reveals individualized fold features

The numerous Ig domains along the titin chain (> 300) share high levels of structural and sequence similarity. Yet, certain Igs have achieved structural and functional differentiation, acting as specific loci for the recruitment of sarcomeric proteins to titin. To reveal whether Ig81 presents individualized features that could support its specific role in CARP binding, we elucidated its atomic structure using X-ray crystallography (Table 1).

The structure of Ig81 reveals a classical Ig-I fold, characteristic of domains from titin. However, Ig81 has a divergent BC-loop at the N-terminal pole of its fold that does not conform with the canonical features of either of the Ig subtypes of titin: the ‘N-conserved’ and ‘N-variable’ types [48]. The ‘N-conserved’ type is characterized by the presence of a long FG β -hairpin hosting a NxxG motif, the prevalence of a PP motif within the N-terminal β -strand A, and a modestly long BC-loop rich in proline residues. The correlated conservation of these three attributes derives from their tight mutual packing at the N-terminal pole of the fold [48]. By contrast, the ‘N-variable’ Ig subtype is defined by shortened BC and FG loops that do not pack against each other [48]. The loop structure is highly conserved within each Ig subtype (Data S7), with the divergence resulting from identifiable sequence differences that alter both the length and composition of these loops in each subtype. Ig81 shows features typical of the ‘N-variable’ subtype (presenting a short FG β -hairpin) but it displays an unusually long BC-loop—these unique characteristics do not conform to either subtype (Fig. 6; Data S7). The protruding BC-loop in Ig81 is accessible, being a good candidate for the mediation of interactions. Thus, it is conceivable that

Fig. 5. SAXS data and *ab initio* models of the CARP/titin complex and its components. (A) Experimental scattering is displayed as dots with error bars, while the curves computed from the *ab initio* models are given as continuous lines; (B) Distance distribution functions; (C) *ab initio* sphere models. Bringing confidence to the calculations, it can be observed that shapes of larger constructs reproduce and expand the features of the smaller segments. Comparison of UN2A and UN2A-Ig81 envelopes suggest that UN2A has an N-terminal protrusion that contributes to its acutely elongated shape. The extension on the right side of UN2A-Ig81 fits an Ig domain within. The envelope of the CARP : UN2A-Ig81 complex shows a transversal enlargement, whose dimensions agree with those of the I-TASSER model of CARP¹⁰⁶⁻³¹⁹ that fits within; (D) Schematic interpretation of the CARP : UN2A-Ig81 complex.

this loop, which is located at the UN2A-Ig81 interface, might play a role in the recruitment of CARP to this locus of titin.

Finally, we explored whether the elongated BC-loop in Ig81 affected the stability of its fold. We derived the T_m of Ig81 by thermal unfolding monitored by DSF, which yielded a value of 67 °C (Data S3). This value indicated that Ig81 has a robust fold. The high T_m of Ig81 correlates with the thermostability of UN2A, and together with SAXS data, suggest

that the UN2A-Ig81 doublet is a structurally stable locus of titin.

Discussion

N2A is a small linker located at the junction of the two main springs of titin: the poly-Ig tandems that extend at low force and the PEVK-rich region that extends at high force [1 and references within]. The current view is that N2A is a largely unstructured region that extends upon

Table 3. Molecular parameters calculated from scattering data.

	MM _{calc} (kDa)	MM _{exp} (kDa)	R_g (Å)	D_{max} (Å)	χ^2
UN2A	13.3	13.0	21.71 ± 0.11	90	1.38
UN2A-Ig81	23.6	19.0	27.06 ± 0.19	120	1.28
Complex	47.7	37.5	27.87 ± 0.22	108–112 ^a	1.14

MM, R_g , D_{max} denote the molecular mass, radius of gyration, and maximal particle size, respectively. MM_{calc} is the theoretical MM of the samples calculated from sequence data. χ^2 is the discrepancy between the experimental curve and those computed from *ab initio* sphere models.

^aIn the CARP : UN2A-Ig81 complex, the protrusions are proportionally smaller than the bulkier core fold so their contribution to the $P(r)$ function in the form of a tail is also relatively smaller. We tested the veracity of the tail in the $P(r)$ function by exhaustively evaluating global fits and *ab initio* models for D_{max} values in the range 100–112 Å. Larger D_{max} values (108–112 Å) yielded best results and *ab initio* models with more interpretable features.

stretch, generating passive force. Unexpectedly, our data indicate that UN2A has an α -helical 3D-fold of anisometric characteristics, which forms a structured complex with CARP. It is known that secondary structure elements determine the mechanical unfolding regime of proteins [49,50]. In helices, the main chain hydrogen bonds align with the helical axis that, in turn, commonly aligns with the force vector during pulling. As a result, helices undergo longitudinal shearing, so that their unfolding requires a force equivalent to the force needed to rupture one bond multiplied by the number of bonds in the helix. Thereby, helical motifs behave as constant force springs, displaying a force plateau while unfolding. This is contrary to entropic springs in which the force rises with extension (following Hooke's law) [51–53]. Thus, helices work in a truly elastic regime, undergoing rapid force-induced folding–refolding transitions, where little energy is dissipated so

Fig. 6. Crystal structure of Ig81. (A) Cartoon structures of representative Ig subtypes in titin: I1 belongs to ‘N-conserved’ subtype and I94 to the ‘N-variable’ [48]. The subtypes have a different loop structure at their N-terminal pole. I81 has mixed features, with the extended conformation of its longer BC-loop given by its packing against the N-terminal β -strand A; (B) Sequence alignment of a selected set of Ig from titin. The ‘N-conserved’ group is highlighted in blue. The β -strand composition of Ig81 is indicated in green. (The BC-loop and FG β -hairpin are indicated in A and B). Interestingly, Ig83 in the titin N2A region, which is truncated in *mdm* mice [9], has an unusual feature somewhat comparable to that of Ig81; namely, an extended FG β -hairpin.

that the complete mechanical energy absorbed in the stretching cycle is released during relaxation. We speculate that the UN2A segment might act as a constant force spring that serves as a mechanical buffer in the sarcomeric I-band.

The stretch response of titin N2A is regulated by CARP binding. Yet, CARP shuttles from its sarcomeric localization in the I-band to the nucleus upon mechanical stress [7]. The response to stretch of CARP together with its ability to act as nuclear transcriptional regulator [12,13] point to a role as possible translator of mechanical signals into gene expression processes in muscle [7]. Our data suggest that CARP mechanosensing could result from the dual domain nature of the UN2A-Ig81-binding site. In such shared binding sites, an alteration of the twist or hinge angles between domains by stretch could sterically alter the affinity of the titin chain for the binding protein, thereby making the interaction mechanically sensitive [54]. In this respect, CARP : UN2A-Ig81 is reminiscent of another mechanosensory complex of titin, that of the E3 ubiquitin ligase MuRF1 with the tandem A168-A169-A170-TK in the sarcomeric M-line [55–57]. Like CARP : UN2A-Ig81, MuRF1 requires at least two titin domains for binding: A168-A169 [55], with no single domain able to bind MuRF1 in isolation [57]. As in Ig81, Ig domain A169 has individualized features: a unique loop between β -strands A and A'. The mutagenesis of this loop abolished MuRF1 binding [56]. These shared features indicate that titin might use a general molecular strategy in the formation of sensory complexes. As a whole, findings from this work call now for an assessment of the mechanical resilience of UN2A and the impact on sarcomere performance of its binding to CARP.

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Author contributions

OM, JRF, and JB conceived and supervised the study; TZ, JRF, BF, JB, IB, and DJR designed and performed experiments and analyzed data; SL provided materials and scientific support; OM, TZ, and JB wrote the manuscript; all authors made manuscript revisions.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Data S1. Size exclusion chromatograms and SDS/PAGE of recombinant samples of CARP and titin N2A domain components produced in this study.

Data S2. SEC-MALLS measurements of CARP and N2A components.

Data S3. Differential Scanning Fluorimetry measurements on CARP and Ig81.

Data S4. NMR analysis indicates that UN2A has a helically folded structure.

Data S5. Comparison of gel filtration chromatograms of UN2A and Ig81.

Data S6. CD spectrum of UN2A upon thermal incubation at 99 °C for 10 min.

Data S7. Comparison of loop structure in Ig subtypes in titin.